Titel der Arbeit:
The contribution of steroids and prostaglandins to the lifespan of *corpora lutea* in domestic cats and lynxes

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The research presented in this cumulative dissertation is based on experiments performed from 01.07.2012 – 31.08.2015 at the Leibniz Institute for Zoo and Wildlife Research in the Department of Reproduction Biology within the *Lynx Research Project*. The dissertation contains a comprising introduction and discussion of three publications in peer-reviewed scientific journals and one manuscript submitted for publication:

Article 1: **Zschockelt L**, Amelkina O, Siemieniuch MJ, Koster S, Jewgenow K, Braun BC. Corpora lutea of pregnant and pseudopregnant domestic cats reveal similar steroidogenic capacities during the luteal life span. J Steroid Biochem Mol Biol. 2014 Oct;144 Pt B:373-81. doi: 10.1016/j.jsbmb.2014.08.010. Epub 2014 Aug 17.
http://www.sciencedirect.com/science/article/pii/S0960076014001897

Article 2: **Zschockelt L**, Amelkina O, Koster S, Painer J, Okuyama MW, Serra R, Vargas A, Jewgenow K, Braun BC. Comparative analysis of intraluteal steroidogenic enzymes emphasises the functionality of fresh and persistent corpora lutea during pro- and metoestrus in the lynx. J Steroid Biochem Mol Biol. 2015 Nov;154:75-84. doi: 10.1016/j.jsbmb.2015.07.001. Epub 2015 Jul 10.
http://www.sciencedirect.com/science/article/pii/S0960076015300145?np=y

Article 3: **Zschockelt L**, Amelkina O, Siemieniuch MJ, Kowalewski MP, Dehnhard M, Jewgenow K, Braun BC. Contribution of luteal prostaglandin synthesis and reception to lifespan of feline corpora lutea. *currently under review in Reproduction*

Article 4: Siemieniuch MJ, Jursza E, Szóstek AZ, **Zschockelt L**, Boos A, Kowalewski MP. Placental origin of prostaglandin F2α in the domestic cat. Mediators Inflamm. 2014;2014:364787. doi: 10.1155/2014/364787. Epub 2014 Feb 11.
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Zusammenfassung

Der Iberische Luchs (*Lynx pardinus*) wird von der IUCN als *stark gefährdet* eingestuft. Neben Maßnahmen zur *in-situ* Erhaltung wurde 2002 das *Ex-situ Iberian Lynx Conservation Breeding Programme* mit dem Ziel der Auswilderung von Luchsen initiiert. Als interdisziplinäres Forschungsinstitut wurde das Leibniz-Institut für Zoo- und Wildtierforschung zu einem wichtigen wissenschaftlichen Partner des Programms und untersucht im Rahmen des *Lynx Research Projects* die Reproduktionsbiologie und Maßnahmen der assistierten Reproduktion. Iberische Luchse zeigen einen saisonalen Monoöstrus und physiologisch persistierende *corpora lutea* (perCL, Gelbkörper), die eine erneute Induktion einer Ovulation außerhalb der Zuchtsaison durch konstant erhöhte Progesteron-(P4)-Plasmawerte verhindern. Nach der Ovulation findet man neben den frisch gebildeten CL (freshCL) funktionell aktive perCL früherer Jahre. Eine erfolglose Verpaarung resultiert im Verlust einer gesamten Zuchtsaison, was bei genetisch wertvollen Luchsen im Programm sehr problematisch ist.

Kenntnis über die artspezifische Ovarphysiologie und den Lebenszyklus der CL sind Voraussetzung, um geeignete Techniken zur Ovulationsinduktion beim Iberischen Luchs zu entwickeln. Da der Zugang jedoch streng limitiert ist, müssen viele Untersuchungen stellvertretend am nahverwandten Eurasischen Luchs (*Lynx lynx*) und auch an Hauskatzen (*Felis silvestris f. catus*) durchgeführt werden. Ziel der Dissertation war es, sich mit der Synthese und Rezeption von Steroiden und Prostaglandinen (PG) in Gelbkörpern von Feliden zu beschäftigen, um Unterschiede in der hormonellen Regulation zwischen Hauskatzen und den beiden europäischen Luchsarten herauszuarbeiten. Die Identifikation potentiell luteotropher und luteolytischer Faktoren im trächtigen und nicht-trächtigen Zyklus, aber auch in fresh- und perCL, soll die Erarbeitung von Protokollen zur artifiziellen Ovulationsinduktion unterstützen.

Katzen zeigen einen saisonalen Polyöstrus. Nach der Ovulation werden CL gebildet, deren Lebensspanne in Abhängigkeit von einer Trächtigkeit durch unterschiedliche P4-Plasmaprofile charakterisiert ist. Deshalb wurden Unterschiede zwischen trächtigen und nicht-trächtigen CL hinsichtlich der steroidogenen Kapazität angenommen. Dem Phänomen der jahrelangen CL-Persistenz bei Luchsen könnten ebenfalls unterschiedliche biochemische Kapazitäten der Gelbkörper zugrunde liegen, wobei intralutealen Steroiden eine luteotrophe Wirkung zugeschrieben wird. Ein natürlicher Östrus während der Zuchtsaison, trotz funktioneller perCL, könnte hingegen auf eine zeitlich begrenzte Reduktion der steroidogenen CL-Aktivität beim Luchs zurückgeführt werden.

In **Artikel 1** und **2** wurde daher die Steroidbiogenese in CL von trächtigen und nicht-trächtigen Katzen sowie in fresh- und perCL von Luchsen im Proöstrus (vor der Verpaarung) und Metöstrus (nach der Verpaarung) untersucht. Es wurde gezeigt, dass während der Gelbkörperphase der Katze CL gleicher Histomorphologie, unabhängig vom Vorhandensein einer Trächtigkeit, ähnliche steroidogene Kapazitäten aufweisen. Die Abnahme der CL-Funktion im Laufe der Gelbkörperphase spiegelt sich in einem graduellen Verlust der *de novo* Steroidbiogenese wider. Bei Luchsen ist die steroidogene Kapazität von perCL zwar im Proöstrus herabgesetzt, jedoch ist die *de novo* Steroidbiogenese im Metöstrus wieder verstärkt. Dieses ist wahrscheinlich auf den Einfluss extralutealer luteotropher Faktoren, die mit dem neuen Zyklus assoziiert sind, zurückzuführen.

Basierend auf diesen Ergebnissen wurde in **Artikel 3** ein potentieller Einfluss von PG des Gelbkörpers auf die Funktion des CL im trächtigen und nicht-trächtigen Zyklus der Katze untersucht. Zudem wurde der Einfluss von PG auf die CL-Persistenz beim Luchs und auf die kurzzeitige Reduzierung der CL-Funktion im Proöstrus analysiert. Die Ergebnisse zeigten, dass die Synthesekapazität und Rezeption von PGE_2 bei Katze und Luchs unabhängig vom CL-Stadium und dem Reproduktionszyklus sind. Hohe Werte an luteotrophem PGE_2 in perCL könnten für die funktionelle und strukturelle CL-Persistenz beim Luchs verantwortlich sein. Der feline CL ist zwar zur Bindung des luteolytischen $PGF_{2\alpha}$ fähig, jedoch ist die Kapazität zur Synthese von $PGF_{2\alpha}$ begrenzt. Daher scheint $PGF_{2\alpha}$ aus dem CL nicht die Regression des Gelbkörpers bei der Katze und ebenfalls nicht die reduzierte CL-Funktion im Proöstrus bei Luchsen zu induzieren. Eine extraluteale Quelle für $PGF_{2\alpha}$ ist sehr wahrscheinlich.

Bei allen Feliden steigt der PGFM-($PGF_{2\alpha}$ Metabolit)-Wert im letzten Drittel der Trächtigkeit an, weshalb die Plazenta als extralutealer Syntheseort für das luteolytische $PGF_{2\alpha}$ in Frage kommt. In **Artikel 4** wurde die Fähigkeit der Plazenta zur Synthese von $PGF_{2\alpha}$ in Abhängigkeit von der Trächtigkeitsdauer bei Katzen analysiert. Die Höchstwerte an $PGF_{2\alpha}$ in der Plazenta wurden, wie auch im Plasma (PGFM), im letzten Trächtigkeitstrimester gemessen. Demnach produziert die Plazenta der Katze $PGF_{2\alpha}$, welches die CL-Regression und Geburt am Ende der Trächtigkeit ermöglicht.

Corpora lutea von Katzen und Luchsen zeigen ähnliche Kapazitäten zur Synthese und Rezeption von Steroiden und PG. Die steroidogene Kapazität ist mit verschiedenen CL-Stadien und dem Reproduktionszyklus der Katze assoziiert und ist am höchsten während der *Bildung* und *Aufrechterhaltung* des CL. Die konstante Synthese und Rezeption von PGE_2 scheint mit dem Erhalt der CL-Funktion gekoppelt zu sein. Feliden weisen keine $PGF_{2\alpha}$-assoziierte luteale

Regression in Abwesenheit einer Trächtigkeit auf, jedoch spielt in der Plazenta gebildetes $PGF_{2\alpha}$ eine luteolytische Rolle. Weitere Faktoren, die für den Abbau bzw. die Persistenz des felinen CL verantwortlich sind, müssen untersucht werden. Dadurch könnte geklärt werden, warum Luchsgelbkörper nur eine transitorische Reduktion der Funktion durchlaufen, jedoch langfristig strukturell und funktionell erhalten bleiben, während CL der Katze einem funktionellen und strukturellen Abbau unterliegen.

Schlüsselwörter:

Corpus luteum, Plazenta, Steroidbiogenese, Prostaglandinsynthese, Feliden, Naturschutz

Summary

The Iberian lynx (*Lynx pardinus*) is declared as *endangered* by the IUCN. Next to *in-situ* conservation approaches, the *Ex-situ Iberian Lynx Conservation Breeding Programme* was initiated in 2002 to reintroduce lynxes into the wild. As an interdisciplinary research institute, the Leibniz Institute for Zoo and Wildlife Research became an important scientific partner of the programme and investigates the reproduction biology and assisted reproduction techniques within the scope of the *Lynx Research Project*. Iberian lynxes exhibit a seasonal monooestrus and physiologically persistent *corpora lutea* (perCL), which prevent a second induction of ovulation outside the breeding season through constantly elevated plasma progesterone (P4) levels. After ovulation, freshly formed CL (freshCL) coexist with functionally active perCL of previous years. Unsuccessful mating results in the loss of a whole breeding season, which in case of genetically valuable lynxes is highly problematic for the programme.

Knowledge on the species-specific ovarian physiology, and especially the life cycle of CL, is an essential prerequisite to develop suitable techniques of ovulation induction for the Iberian lynx. Because access to Iberian lynxes is strictly limited, many investigations need to be conducted on the closely related Eurasian lynx (*Lynx lynx*) and the domestic cat (*Felis silvestris f. catus*). The aim of the dissertation was to characterise the synthesis and reception of steroids and prostaglandins (PGs) in CL of felids to elucidate differences in the hormonal regulation between domestic cats and the two European lynx species. The identification of potential luteotrophic and luteolytic factors of the pregnant and non-pregnant cycle, but also in fresh and perCL, should support the establishment of protocols for artificial ovulation induction.

Domestic cats exhibit a seasonal polyoestrus. After ovulation, CL are built with lifespans characterised by different plasma P4 profiles dependent on pregnancy. Therefore, differences in CL of pregnant and non-pregnant cycles, regarding the steroidogenic capacity, were assumed. The phenomenon of long term CL persistence in lynxes might underlie different biochemical capacities of the CL as well, whereby intraluteal steroids might exert a luteotrophic role. Occurrence of a natural oestrus during the breeding season, despite functional perCL, might rather point to a temporary reduction of the steroidogenic CL activity in lynxes.

In **Article 1** and **2** steroid biogenesis pathways were analysed in CL of pregnant and non-pregnant cats and in fresh and perCL of lynxes at prooestrus (pre-mating) and metoestrus (post-mating). It was shown that during the luteal lifespan of the cat, CL of equal histomor-

phological stages exhibit similar steroidogenic capacities, irrespectively of an ongoing pregnancy. The functional demise of CL during the luteal phases mirrors the gradual loss of *de novo* steroid biogenesis. In lynxes, the steroidogenic capacity is temporarily limited at prooestrus, but *de novo* steroid biogenesis is again enhanced during metoestrus. This is most likely based on the influence of external luteotrophic factors associated with the new cycle.

Based on these findings, in **Article 3** the potential influence of luteal PGs on the CL function during the pregnant and non-pregnant cycle in cats was analysed. Furthermore, a role of PGs to maintain CL persistence in lynxes and to transiently reduce the function of CL at prooestrus was investigated. The results indicated that the capacity for synthesis and reception of PGE_2 in cat and lynxes is independent on the CL stage and reproductive cycle. High levels of luteotrophic PGE_2 in perCL might be responsible for the functional and structural CL persistence in lynxes. The feline CL is capable of binding luteolytic $PGF_{2\alpha}$; however, the capacity to synthesise $PGF_{2\alpha}$ is limited. Thus, luteal $PGF_{2\alpha}$ neither induces regression of the cat CL, nor the reduced CL function in prooestrous lynxes. An extraluteal source of $PGF_{2\alpha}$ is most likely.

All felids show increased PGFM ($PGF_{2\alpha}$ metabolite) levels in the last third of pregnancy, wherefore the placenta was suggested as an extraluteal source for production of luteolytic $PGF_{2\alpha}$. In **Article 4**, the capacity of the feline placenta to synthesise $PGF_{2\alpha}$ dependent on the duration of pregnancy was determined in cats. Peak levels of placental $PGF_{2\alpha}$, similarly to plasma PGFM, were measured during the last trimester of pregnancy. Therefore, the placenta synthesises $PGF_{2\alpha}$, enabling CL regression and parturition at the end of pregnancy in cats.

Corpora lutea of cat and lynxes exhibit similar capacities to synthesise and respond to steroids and PGs. The steroidogenic capacity is associated with different CL stages and the reproductive cycle, being highest during the CL *formation* and its *maintenance* in the cat. The constant production and reception of PGE_2 seems to be linked with maintaining CL function. Felids show no $PGF_{2\alpha}$-associated luteal regression in the absence of pregnancy, but $PGF_{2\alpha}$ originating from the placenta exhibits a likely luteolytic role. Other factors causing the regression, respectively the persistence, of the feline CL need to be investigated, to explain why CL of lynxes experience a transitory reduction of their function, while preserving their structural and functional integrity, whereas cat CL undergo functional and structural regression.

Keywords:
corpus luteum, placenta, steroid biogenesis, prostaglandin biosynthesis, felids, conservation

Abbreviations

AKR	aldo-keto reductase
CA	*corpus albicans*
CL	*corpus luteum*
CYP	cytochrome *P*-450 oxidase
D/M	development/maintenance
E2	oestradiol
EIA	Enzyme-Linked Immunosorbent Assay
EL	Eurasian lynx
ER	early regression
EU-LIFE	Lince Andalucia - Population Recovery of Iberian Lynx in Andalusia
F	formation
fresh	freshly formed; *i.e.*, after ovulation
HSD	hydroxysteroid dehydrogenase
IH	immunohistochemistry
IL	Iberian lynx
ILCBP	Iberian Lynx Conservation Breeding Programme (Messines, Portugal; Huelva, Spain)
IUCN	International Union for Conservation of Nature and Natural Resources
IZW	Leibniz Institute for Zoo and Wildlife Research (Berlin, Germany)
LR	late regression
P4	progesterone
PCR	polymerase chain reaction
per	persistent; *i.e.*, of previous years
PG	prostaglandin
PGFM	$PGF_{2\alpha}$ metabolite
PGFS	$PGF_{2\alpha}$ synthase
PP	pseudopregnancy; *i.e.*, non-pregnant luteal phase
PR	pregnancy
PTGER	prostaglandin E receptor
PTGES	PGE_2 synthase
PTGFR	prostaglandin F receptor
PTGS/COX	prostaglandin endoperoxide G/H synthase/cyclooxygenase
qPCR	quantitative polymerase chain reaction
SNO	Statens Naturoppsyn (Trondheim, Norway)
SRD	5α-steroid reductase
STAR	steroidogenic acute regulatory protein
SVA	National Veterinary Institute (Uppsala, Sweden)
Vetanat	Institute of Veterinary Anatomy (Zurich, Switzerland)
WB	Western Blot
ZIPR	Institute of Animal Reproduction and Food Research (Olsztyn, Poland)

Introduction

Conservation efforts to prevent extinction of the Iberian lynx

Lynxes are medium sized carnivores of the family Felidae. The genus *Lynx* currently consists of four species: the bobcat (*Lynx rufus* Schreber, 1777) and the Canada lynx (*L. canadensis* Kerr, 1792) in North America and the Eurasian (*L. lynx* Linnaeus, 1758) and the Iberian lynx (*L. pardinus* Temminck, 1827) in Eurasia. The genus *Lynx* descends from a common ancestor that diverged from other felid species more than 6 million years ago (Johnson *et al.* 2004). The Eurasian lynx, the Canada lynx and the bobcat are considered as *least concern* by the International Union for Conservation of Nature and Natural Resources (IUCN). Until lately, the Iberian lynx, exclusively found on the Iberian Peninsula (Palomares *et al.* 2005), was the only felid species listed as *critically endangered* (Simon *et al.* 2012). Due to habitat loss, increased fragmentation of populations, and because of a drastic reduction of rabbits, the main prey species, approximately 200 individuals were left in the wild in 2002 (Johnson *et al.* 2004). Consequently, an integrated species conservation management plan, which links the *in-situ* conservation efforts of the *Lince Andalucia - Population Recovery of Iberian Lynx in Andalusia Project* (EU-LIFE) with the *Ex-situ Iberian Lynx Conservation Breeding Programme* (ILCBP), was started to prevent extinction of the Iberian lynx. As a direct effect, the Iberian lynx was declared as *endangered* in 2015 (IUCN 2015). Beside efforts to reconstruct habitats and ensure sustainable rabbit populations, captive breeding was considered a new and important part of the programme to support and restore wild populations (Palomares *et al.* 2010). Before 2005, captive breeding of Iberian lynxes was never achieved (Vargas *et al.* 2008).

The captive breeding programme offers the opportunity to perform basic reproductive research and to apply assisted reproduction techniques (ARTs). In this regard, sperm collection and cryopreservation (Ganan *et al.* 2010) and artificial insemination can be applied if natural breeding fails and to allow gene flow between separated populations (Swanson *et al.* 2007). However, regular endocrine and ultrasound examinations revealed that female lynxes exhibit a reproductive peculiarity. Physiological persistent *corpora lutea* (perCL) hinder the induction of another cycle after the breeding season (Göritz *et al.* 2009, Painer *et al.* 2014). Thus, the reproductive potential of genetically valuable lynxes is lost for one year if mating fails (Jewgenow *et al.* 2014, Painer *et al.* 2014). Knowledge about the female reproductive cycle is fundamental for the integrated species conservation plan (Palomares *et al.* 2005), but the mechanisms underlying physiological persistence of CL in lynxes remain unclear.

The ILCBP provides the basis for research on the reproduction physiology and endocrinology with a main focus on non-invasive pregnancy diagnosis (Vargas *et al.* 2008), since other studies on Iberian lynxes are strictly limited by their conservation status. Therefore its close relative, the less endangered Eurasian lynx, is commonly used to investigate the reproduction biology of lynxes (Göritz *et al.* 2009, Painer *et al.* 2014). Due to the phylogenetic proximity, research on the function and regression of the feline CL, and thus the regulation of the ovarian cycle, is also performed on the domestic cat (*Felis silvestris f. catus* Linnaeus, 1758), to develop ARTs, which later can be applied to endangered felid species (Wildt *et al.* 2001, Pelican *et al.* 2006). Being the scientific partner of the ILCBP, the Leibniz Institute for Zoo and Wildlife Research (IZW) initiated the *Lynx Research Project* compiling reproductive research on domestic cats, Eurasian lynxes and, if possible, on Iberian lynxes. As an interdisciplinary research institute, the IZW is dedicated to evolutionary wildlife research for conservation interventions. The consolidated knowledge about specific reproductive adaptations will help to improve the reproduction potential of endangered lynxes.

Formation, maintenance and regression of the *corpus luteum*

The main function of the CL is secretion of the sex steroid hormone progesterone (P4) (Niswender *et al.* 2000), required for regulation of the ovarian cycle (McCracken *et al.* 1999) and the establishment and maintenance of pregnancy (Bowen-Shauver & Telleria 2003). The transient ovarian endocrine gland is formed from the ovulated follicle by luteinisation of follicular cells, endothelial cell invasion, and tissue remodelling (Stocco *et al.* 2007). After a preovulatory surge of luteinising hormone (LH) from the anterior pituitary, ovulation of the mature follicle is induced (Concannon *et al.* 1980). During luteinisation, residual follicular granulosa and theca cells differentiate and form the CL (Niswender *et al.* 2000). The developed CL consists of a heterogeneous cell population with two types of steroidogenic luteal cells. The cells derived from granulosa cells are large luteal cells (LLC), and those from theca cells are small luteal cells (SLC) (McCracken *et al.* 1999). In addition, the CL consists of endothelial cells, fibroblasts, pericytes, and cells of the immune system (Stocco *et al.* 2007).

The functional CL lifespan varies due to different reproductive events, such as the ovarian cycle, pregnancy, pseudopregnancy or lactation (Bowen-Shauver & Telleria 2003). Different reproductive strategies, like monooestrous or polyoestrous reproductive patterns, are related to the duration of the luteal phase as well (Göritz *et al.* 2009, Brown 2011). Shortened luteal phases were documented in pseudopregnant rabbits (Horrell *et al.* 1972) and rats (Pepe & Rothchild 1974), whereas in mink (Allais & Martinet 1978) and ferret (Heap & Hammond

1974), luteal phases of pregnant and pseudopregnant females reveal similar lengths. The luteal phases of pregnant and non-pregnant dogs proceed similarly until shortly before parturition (Concannon *et al.* 1989). Subsequently, CL of non-pregnant dogs can retain their function exceeding the time of parturition (Concannon 1993, Nohr *et al.* 1993). In rats, the CL can persist for several cycles, although they are not fully functional (Davis & Rueda 2002) and in cows, *postpartum* persistence of CL usually is associated with uterine disorders (Magata *et al.* 2012). Luteotrophins assumed to maintain CL function in mammals, among others, include LH and the follicle stimulating hormone (FSH) (Wildt *et al.* 1980, Beindorff & Einspanier 2010), the steroid hormones oestradiol (E2) and P4 (Musah *et al.* 1990, Tsutsui & Stabenfeldt 1993), prostaglandins (PGs) (Kowalewski *et al.* 2013), as well as the peptide hormones prolactin and relaxin (Banks *et al.* 1983, Addiego *et al.* 1987).

At the end of the luteal phase, CL enter a process of functional and structural regression (Bowen-Shauver & Telleria 2003). Functional regression comprises the loss of the capacity to synthesise P4 (McGuire *et al.* 1994, Hoyer 1998), whereas structural regression includes involution of the luteal tissue, concomitant to programmed apoptotic cell death (Tilly 1996, Bowen-Shauver & Telleria 2003). The conserved role of $PGF_{2\alpha}$ in mediating luteal regression is generally accepted (Niswender *et al.* 2000, Arosh *et al.* 2004). In most of the species studied so far, $PGF_{2\alpha}$ induces structural luteal regression by activating apoptotic pathways (Davis & Rueda 2002, Yadav *et al.* 2005). Apoptosis-related factors of the intrinsic apoptotic pathway include members of the caspase (CASP) protein family like CASP3 (Khan *et al.* 2000, Peluffo *et al.* 2007), which initiate the final execution phase of apoptosis. Anti-apoptotic, pro-survival factors of the B-cell CLL/lymphoma 2 (Bcl-2) family, such as BCL2, can protect cells from apoptosis (Reed 1994, Korsmeyer 1999).

The seasonal polyoestrus of the domestic cat

Domestic cats exhibit a seasonal polyoestrus, *i.e.*, during the breeding season, extending from early spring to summer in moderate latitudes (Fabian & Preuss 1966), cats exhibit several oestrus cycles (Figure 1). At prooestrus (period before breeding season, pre-mating), FSH induces ovarian folliculogenesis (Shille *et al.* 1979). During oestrus (breeding season, period of sexual receptivity), an anovulatory cycle can occur, which is followed by an interoestrus interval (Arbeiter 1994). Different to that, ovulation can be induced by coital, or an adequate, stimulation and occurs 1–2 days *post coitum* (*p.c.*) (Concannon *et al.* 1980, Wildt *et al.* 1981) or, in few cases, spontaneously without cervical or vaginal stimulation (Lawler *et al.* 1993, Pelican *et al.* 2005), preceded by a preovulatory LH surge (Shille *et al.* 1979).

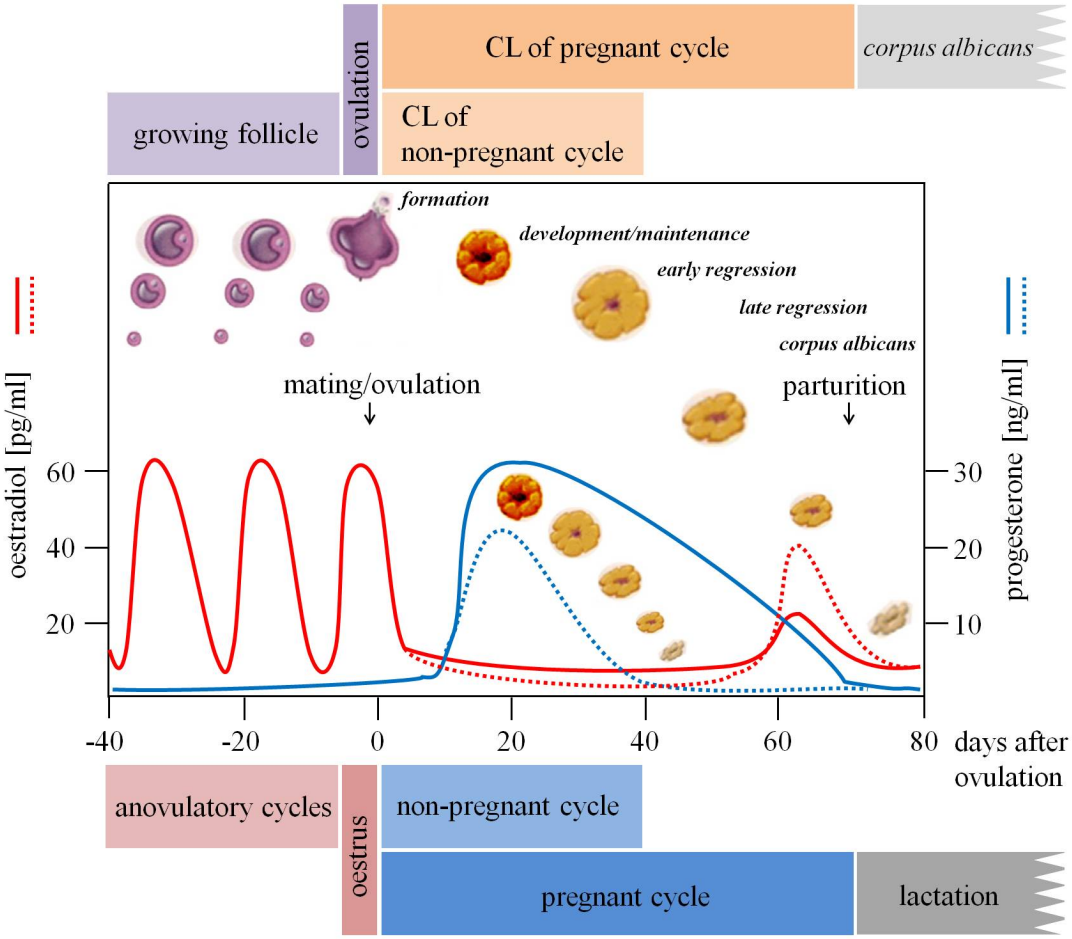

Figure 1: Scheme of the domestic cat reproductive cycle (seasonal polyoestrus). Plasma oestradiol (E2) and progesterone (P4) profiles are depicted in relation to ovarian structures, luteal phases and reproductive cycles. Cats either exhibit anovulatory cycles, or ovulation is induced or occurs spontaneously at the end of the follicular phase. The follicle ruptures and releases the oocyte. The ovulation is followed by the formation of *corpora lutea* (CL) during the luteal phases of pregnant or non-pregnant cycles. Until days 12–14 *post coitum* (*p.c.*), plasma P4 levels increase similarly. Thereafter, P4 levels are substantially higher in pregnant luteal phases until days 25–30 *p.c.* and constantly decrease until basal levels at parturition. During the non-pregnant cycle, P4 reaches peak values at days 25–30 *p.c.*, but already becomes basal at days 25–40 after ovulation. The end of the luteal lifespan (prepartal or cyclic luteal regression) is depicted by *corpora albicantia*. A new cycle is initiated or the cat enters the seasonal anoestrus. Dotted lines show profiles of non-pregnant cycles. Modified after (Arbeiter 1994), (Verhage *et al.* 1976) and (Jewgenow *et al.* 2014).

Oestrus is followed by metoestrus (pregnant or non-pregnant luteal phase, post-mating). Pregnancy lasts about 60–66 days (Tsutsui & Stabenfeldt 1993). Formation of CL and the subsequent functional luteal phase are reflected by plasma P4 levels exceeding basal levels of 0.5–2 ng/ml 1–2 days after ovulation (Verhage *et al.* 1976). Progesterone levels rise steadily until days 12–14 *p.c.* (15–20 ng/ml) (Verhage *et al.* 1976), coinciding with the time of blastocyst implantation (Leiser 1979, Boomsma *et al.* 1991). Thereafter, P4 levels reach peak values of 40–60 ng/ml at days 25–30 *p.c.* (Verhage et al. 1976). At day 50 *p.c.*, plasma P4 levels are decreased below 15 ng/ml and reach 4–5 ng/ml at parturition (Verhage *et al.* 1976).

After parturition, P4 values decline below threshold levels of 1 ng/ml (Verhage *et al.* 1976). During prooestrus, plasma E2 levels (< 20 pg/ml) are basal and reach preovulatory peak values (40–60 pg/ml) at oestrus (Verhage *et al.* 1976). Thereafter, E2 levels sharply decline until day 5 *p.c.* (12–20 pg/ml) and remain at basal levels (< 15 pg/ml), until they slightly rise prior to parturition (20–40 pg/ml) (Verhage *et al.* 1976, Schmidt *et al.* 1983). If ovulation is accompanied by infertile mating, it is followed by a shortened non-pregnant cycle (pseudopregnancy). Progesterone rises at similar rates as during pregnancy for the first 12–14 days *p.c.*; differences between both profiles become visible around days 25–30 *p.c.*, when pregnant cats have higher P4 levels (Paape *et al.* 1975, Verhage *et al.* 1976). During the non-pregnant cycle, the peak values are lower (25 ng/ml), and P4 already becomes basal at days 25–40 *p.c*. (Paape *et al.* 1975, Verhage *et al.* 1976). The levels of plasma E2 are in a similar range compared to pregnancy (Paape *et al.* 1975, Verhage *et al.* 1976). At the end of the luteal phase, cats go into seasonal anoestrus (period of sexual inactivity, P4 levels remain at < 0.5 ng/ml), or a new oestrus is initiated.

In a detailed study conducted within the *Lynx Research Project*, Amelkina *et al.* revealed that the CL of cats undergoes severe histomorphological transformations with progression through the luteal stages (Amelkina *et al.* 2015). During *formation*, highly neovascularised CL undergo tissue reorganisation and luteinisation and contain a mixture of predominantly small transforming follicular cells with different degrees of luteinisation. At *development/maintenance*, polyhedral luteal cells are increased in size and exhibit accumulation of numerous lipid droplets on the cell periphery. During *early regression*, CL reveal first signs of luteal regression and large vacuoles are coarsely organised over the cells, whereby the ratio of non-steroidogenic to luteal cells increases. In the *late regression*, CL show two types of extreme cell modifications. Luteal cells of the first type show a heavy degree of large vacuoles that are coarsely scattered in the cell and do not react to lipid staining; nuclei of the small luteal cells are mostly condensed. Luteal cells of the second stage acquire a signet ring form because of extremely large vacuoles, occupying almost the whole cell. The *corpus albicans* is considered the last stage of the CL lifespan and presents highly modified luteal cells with condensed nuclei. Prominently smaller luteal cells become outnumbered by non-steroidogenic cells (Amelkina *et al.* 2015). The CL of lactating cats remains prominent at least until day 63 after parturition (Dawson 1946, Amelkina *et al.* 2015).

The monooestrous reproduction of Eurasian and Iberian lynxes

The reproductive biology of lynxes is rather atypical (Göritz *et al.* 2009) (Figure 2). As the crucial reproductive peculiarity, the Iberian (Palomares *et al.* 2005), the Eurasian (Kvam 1991, Dehnhard *et al.* 2008) and the Canada lynx (Fanson *et al.* 2010) exhibit a strict seasonal monooestrous cycle, which is contrary to the polyoestrous reproductive pattern of most other felids (Brown 2011), including the bobcat (Crowe 1975, Parker & Smith 1983). The breeding season of Iberian lynxes falls into January to February, whereas Eurasian lynxes exhibit oestrus during January to April (Jewgenow *et al.* 2014). Ovulation is induced by mating (Palomares *et al.* 2005, Jewgenow *et al.* 2009), but spontaneous ovulation in captivity occurs as well (Fanson *et al.* 2010, Painer *et al.* 2014). Painer *et al.* conducted detailed longitudinal ultrasound and endocrine examinations in Eurasian lynxes (Painer *et al.* 2014). At prooestrus, plasma P4 is decreased to 2.7 ng/ml from 4.7 ng/ml in dioestrus, whereas $PGF_{2\alpha}$ metabolite (PGFM) levels are increased from 1.8 to 2.6 ng/ml (Painer *et al.* 2014). The oestrus period is accompanied by slightly elevated plasma E2 levels (1.5 ng/ml), whereas P4 levels remain at 2 ng/ml (Painer *et al.* 2014). During metoestrus, elevated plasma P4 levels (> 15 ng/ml) are indicative of CL formation (Painer *et al.* 2014). A fertile mating is followed by pregnancy (66–70 days) and P4 levels increase rapidly and become substantially elevated (up to 84 ng/ml) (Painer *et al.* 2014). Plasma P4 remains high throughout lactation (up to 170 ng/ml) but drops to 5 ng/ml after weaning (Painer *et al.* 2014). According to urinary and faecal samples, PGFM levels increase during the last trimester of gestation, with a prepartal peak. After parturition, the PGFM levels become basal (Finkenwirth *et al.* 2010, Dehnhard *et al.* 2012).

An infertile mating is followed by a non-pregnant luteal phase, in which no changes in the plasma levels of P4, E2 and PGFM occur throughout the year, excluding oestrus (Painer *et al.* 2014). Thus, lynxes do not exhibit a typical anoestrus (Jewgenow *et al.* 2014) and show functional luteal activity outside the breeding season (prolonged dioestrus) with average basal P4 levels of 5 ng/ml (Painer *et al.* 2014). In contrast to several other felid species (Brown *et al.* 1994, Brown *et al.* 2001) non-invasive measurement of faecal and urinary steroid metabolites is inappropriate to distinguish pregnancy from a non-pregnant luteal phase in the Iberian (Pelican *et al.* 2006, Jewgenow *et al.* 2009), Eurasian (Dehnhard *et al.* 2008) and Canada lynx (Fanson *et al.* 2010). In all three lynx species, the physiological persistence of CL was confirmed (Göritz *et al.* 2009, Fanson *et al.* 2010, Carnaby *et al.* 2012, Painer *et al.* 2014). These perCL are preserved structurally for at least two subsequent years (Painer *et al.* 2014).

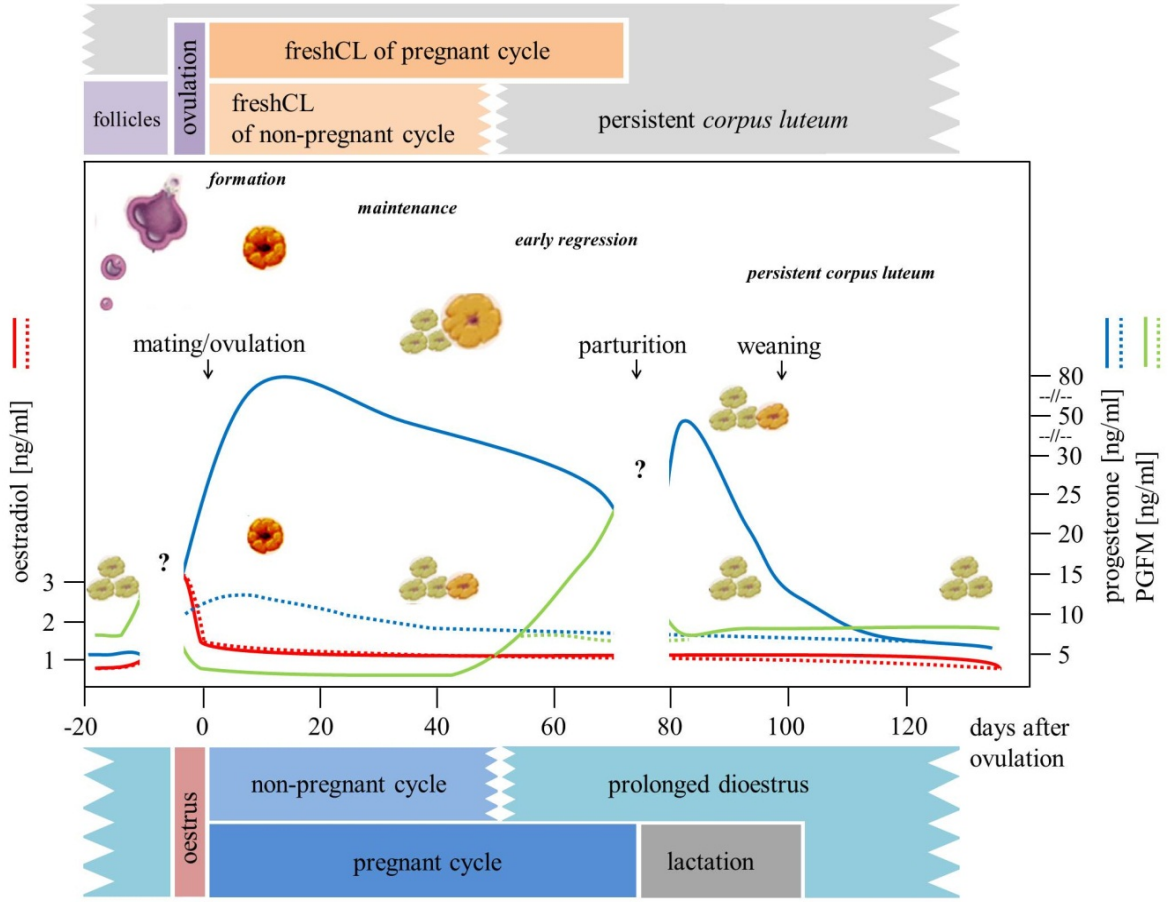

Figure 2: Scheme of the Eurasian lynx reproductive cycle (seasonal monooestrus). Plasma oestradiol (E2), progesterone (P4) and $PGF_{2\alpha}$ metabolite (PGFM) profiles are depicted in relation to ovarian structures, luteal phases and reproductive cycles. At the end of the follicular phase, the follicle ruptures and releases the oocyte. Ovulation is followed by the formation of fresh *corpora lutea* (freshCL). FreshCL coexist with persistent CL of previous years (perCL). At the beginning of the breeding season an elevation of plasma PGFM associated with a P4 decrease is hypothesised (deficient data). During the last trimester of pregnancy, plasma P4 decreases, concomitant to an increase of PGFM. Data on PGFM and P4 levels at parturition are missing. No typical anoestrous period is observed and plasma P4 (on average 5 ng/ml) is detectable throughout the year. Dotted lines indicate profiles of non-pregnant cycles. Modified after (Painer *et al.* 2014) and (Jewgenow *et al.* 2014).

In polyoestrous bobcats, CL of unknown functionality persist morphologically as well (Duke 1949, Crowe 1975). During the breeding season, freshly formed CL of a recent ovulation (freshCL) coexist with perCL from previous years (Carnaby *et al.* 2012, Axner *et al.* 2013, Painer *et al.* 2014). It was hypothesised that physiological persistence of CL is responsible for constantly elevated plasma P4 levels, which in a negative feedback prevent folliculogenesis and ovulation outside the oestrus period, to ensure the monooestrous reproductive pattern (Göritz *et al.* 2009, Painer *et al.* 2014). Irrespectively of constant luteal activity, lynxes enter oestrus and give birth at term, which might be caused by a temporal reduction of luteal function prior to the breeding season and prior to parturition (Painer *et al.* 2014).

Conventional and alternate steroid biogenesis pathways

Stimulation of ovarian follicular and luteal cells by FSH and LH increases mobilisation of cholesterol and synthesis of steroidogenic enzymes (Waterman 1994) (Figure 3). The substrate for *de novo* steroid biogenesis is cholesterol, which is mainly synthesised in the liver and then transported *via* the bloodstream to steroidogenic tissues as circulating plasma low-density (LDL) or high-density (HDL) lipoproteins (Krisans 1996, Niswender *et al.* 2000). Under conditions of lipid deprivation, luteal cells are capable of synthesising cholesterol *de novo* from acetate (Cook *et al.* 1967). Lipoprotein-bound cholesterol is taken up by the cell and is stored as cholesterol esters in lipid droplets of cytoplasmic vacuoles (Miller 2008). The transport of free cholesterol from the outer to the inner mitochondrial membrane is facilitated by the steroidogenic acute regulatory protein (STAR) (Clark *et al.* 1994, Stocco & Clark 1996). Mutations of *STAR* almost completely eliminate steroid biogenesis by adrenals and gonads (Lin *et al.* 1995). The cytochrome *P*-450 cholesterol side-chain cleavage enzyme (CYP11A1) is located on the inner mitochondrial membrane and catalyses the first and rate-limiting enzymatic conversion of cholesterol to pregnenolone (Stone & Hechter 1954). Similar to STAR, deletion of the gene for *CYP11A1* in the rabbit (Yang *et al.* 1993) and mouse (Hu *et al.* 2002) eliminates the capacity for *de novo* steroid biogenesis.

Pregnenolone is converted to P4 by different microsomal and mitochondrial isoforms of 3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerase (HSD3B) (Thomas *et al.* 1989). The isoforms HSD3B1 and HSD3B2 are the only two isoforms involved in the biosynthesis of all active steroids and convert 17α-OH pregnenolone to 17α-OH progesterone, dehydroepiandrosterone (DHEA) to androstenedione, and androstenediol to testosterone with similar K_m and V_{max} values (Payne & Hales 2004, Miller 2008). Pregnenolone and P4 can further undergo 17α-hydroxylation yielding 17α-OH pregnenolone and 17α-OH progesterone catalysed by microsomal cytochrome *P*-450 steroid 17α-monooxygenase (CYP17A1) bound to the endoplasmatic reticulum (Miller 2008). Humane and bovine CYP17A1 further converts 17α-OH pregnenolone to DHEA (Auchus *et al.* 1998) and the rodent enzyme converts 17α-OH progesterone to androstenedione (Payne & Hales 2004). Microsomal cytochrome *P*-450 aromatase (CYP19A1) resides in the endoplasmatic reticulum and aromatises androstenedione and testosterone to oestrone and oestradiol, respectively (Simpson *et al.* 1994, Miller 2008). Different isozymes of 17β-hydroxysteroid dehydrogenases (HSD17Bs) are essential for the final steps of steroid synthesis (Miller 2008).

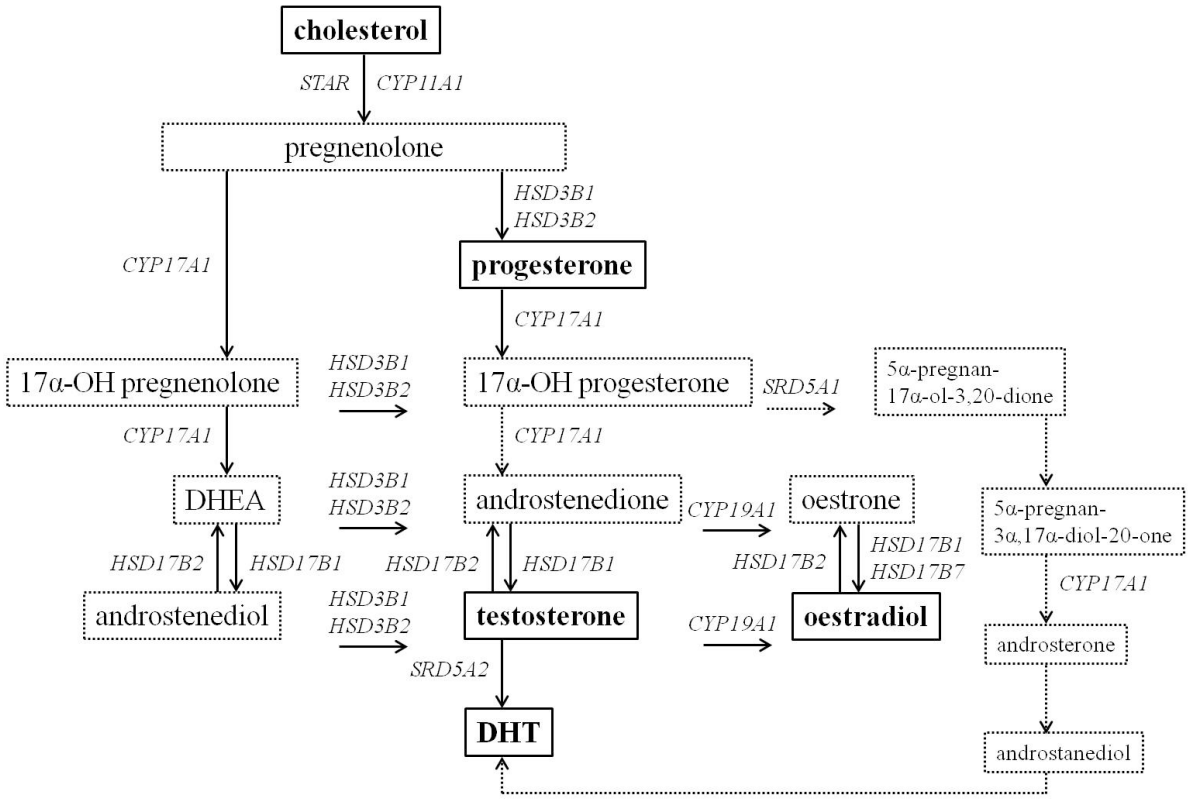

Figure 3: Conventional (–›) and alternate (‹···›) steroid biogenesis pathways of progesterone, oestradiol and testosterone with selected steroidogenic enzymes. Steroidogenic acute regulatory protein (STAR) facilitates the rapid influx of cholesterol to the mitochondria. Mitochondrial cholesterol side-chain cleavage enzyme (CYP11A1) catalyses the conversion of cholesterol to pregnenolone. The 3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerases (HSD3Bs) convert several different Δ^5 steroids to Δ^4 steroids. Qualitative regulation of the steroid produced is determined by microsomal steroid 17α-monooxygenase (CYP17A1). Aromatase (CYP19A1) converts androgens (androstenedione or testosterone) to oestrogens (oestrone or oestradiol). Different isozymes of 17β-hydroxysteroid dehydrogenases (HSD17Bs) are essential for the final steps of steroid synthesis. Two isoforms of 5α-steroid reductases (SRD5As) convert testosterone to dihydrotestosterone (DHT) or, in an alternate pathway, reduce 17α-OH progesterone to yield androstanediol as the direct precursor for DHT. The steroidogenic pathway culminates in synthesis of DHT and oestrogens. Cholesterol and biologically active steroids are highlighted in **bold**, whereas steroid intermediates and metabolites are depicted by doted frames. Associated steroidogenic enzymes are shown in *italics*. Modified according to (Miller 2008), (Ghayee & Auchus 2007) and (Payne & Hales 2004).

In particular, HSD17B1 catalyses conversion of DHEA to androstenediol, androstenedione to testosterone and oestrone to oestradiol (Tremblay *et al.* 1989, Puranen *et al.* 1997, Moghrabi & Andersson 1998), whereas HSD17B2 catalyses the reverse reactions (Miller 2008). In the absence of HSD17B1, oestrone is converted to highly active oestradiol by microsomal HSD17B7 (Peltoketo *et al.* 1999). By 5α-steroid reductase isozyme 2 (SRD5A2), testosterone can be converted to the more potent androgen dihydrotestosterone (DHT) (Ghayee & Auchus 2007, Azzouni *et al.* 2012). In an alternate pathway, 17α-OH progesterone is 5α-reduced by SRD5A1 (Minjarez *et al.* 2001, Auchus 2004) and the yielded androstanediol is used as precursor for synthesis of DHT.

Biosynthesis and reception of prostaglandin E_2 and $F_{2\alpha}$

Luteal cells of most mammalian species possess an inherent capacity to produce PGs (Olofsson & Leung 1994). The CL is rich in arachidonic acid (AA), stored in membrane phospholipids as primary precursor of all PGs (Hansel & Cain 1996) (Figure 4). The first rate-limiting step in PG biosynthesis is liberation of AA from cell membrane phospholipids by cytosolic phospholipase A_2 ($cPLA_2$) (Clark *et al.* 1991). Null mutation of *$cPLA_2$* leads to infertile phenotypes (Bonventre *et al.* 1997). The free AA is converted to PGG_2/PGH_2 by two isoforms of the rate-limiting prostaglandin endoperoxide G/H synthase, also termed cyclooxygenase (PTGS/COX) (Wiltbank & Ottobre 2003). Whereas PTGS1/COX1 is a constitutively expressed form responsible for regulating homeostatic functions, PTGS2/COX2 is generally undetectable in most tissues, but can be induced (Williams *et al.* 1999). Female *PTGS2/COX2* null mice suffer from multiple failures in reproductive processes (Lim *et al.* 1997).

Terminal synthases are responsible for the selective production of active PGs, such as PGE_2 and $PGF_{2\alpha}$ (Fortier *et al.* 2008), wherein three forms of prostaglandin E synthases (PTGES, PTGES2, and PTGES3) catalyse the conversion of PGH_2 to PGE_2 (Smith & Dewitt 1996). The only isoform described so far in the dog CL is PTGES (Kowalewski *et al.* 2008), which is often coupled with inducible PTGS2/COX2 expression (Fortier *et al.* 2008). Prostaglandin $F_{2\alpha}$ is selectively produced by enzymatic conversion of PGH_2 to $PGF_{2\alpha}$ *via* several PGF synthases with 9,11-endoperoxide dehydrogenase activity belonging to the aldo-keto reductase family (*e.g.* PGFS/AKR1B1 and PGFS/AKR1C3) (Fortier *et al.* 2008). The canine-specific PGFS responsible for conversion of PGH_2 to $PGF_{2\alpha}$ in the dog CL is PGFS/AKR1C3 (Gram *et al.* 2013). The enzyme PGFS/AKR1C3 is also capable to convert prostaglandin D synthase (PGDS) derived PGD_2 to $PGF_{2\alpha}$ (Fortier *et al.* 2008). A PGE_2-9-ketoreductase (9KPGR) converts PGE_2 to $PGF_{2\alpha}$ and *vice versa* (Asselin & Fortier 2000).

Plasma PGs are rapidly inactivated in the lungs and the metabolites are cleared in the kidney (Piper *et al.* 1970, Fortier *et al.* 2008). The key enzyme 15-hydroxy prostaglandin dehydrogenase (HPGD) (Kankofer 1999) enzymatically catabolises PGE_2 and $PGF_{2\alpha}$ into biologically inactive 13,14-dihydro-15-keto PGE_2 (PGEM, PGE_2 metabolite) and 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM) (Tai *et al.* 2002). The PG metabolites have a longer half-life and can be used to monitor secretion of short living $PGF_{2\alpha}$ in plasma, urine and faeces (Ginther *et al.* 2007). Local catabolism by HPGD was shown, *e.g.*, in bovine endometrium (Fortier *et al.* 2008), ovine CL (Silva *et al.* 2000) and canine placenta (Gram *et al.* 2013).

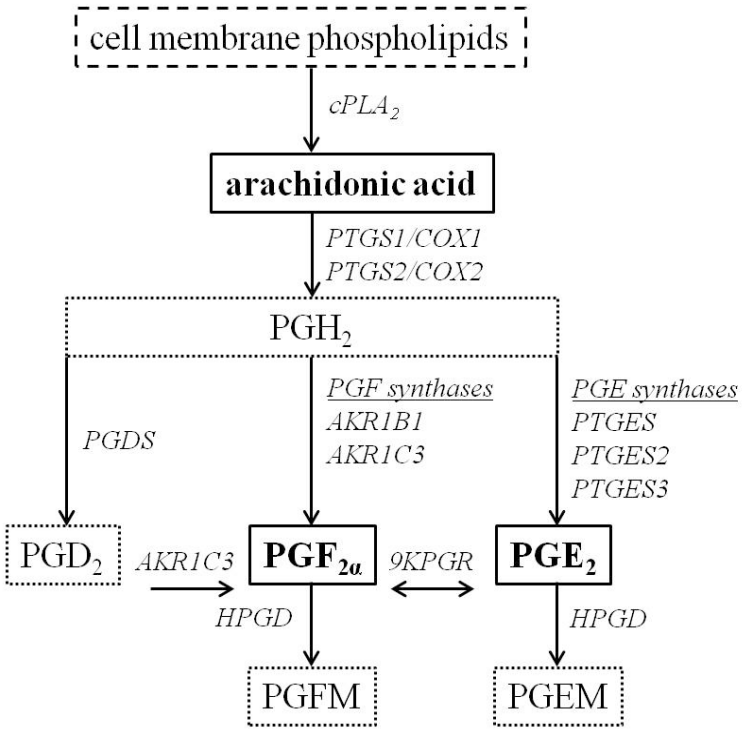

Figure 4: Synthesis and metabolism pathways of prostaglandin E_2 and $F_{2\alpha}$ with selected synthesis enzymes. Cytosolic phospholipase A_2 ($cPLA_2$) releases arachidonic acid (AA) from cell membrane phospholipids. Prostaglandin endoperoxide G/H synthase/cyclooxygenase (PTGS1/COX1, PTGS2/COX2) converts AA into PGH_2, the precursor of all prostaglandins (PGs). Prostaglandin H_2 is converted to PGs by specific synthases. Isoforms of PGE synthases (PTGES, PTGES2, PTGES3) catalyse conversion of PGH_2 to PGE_2. Prostaglandin $F_{2\alpha}$ is produced mainly by conversion of PGH_2 by PGF synthases of the aldo-keto reductase family (PGFS/AKR). The PGD synthase (PGDS) provides PGD_2, which is converted to $PGF_{2\alpha}$ by PGFS/AKR1C3. A PGE_2-9-ketoreductase (9KPGR) utilises PGE_2 for $PGF_{2\alpha}$ synthesis and *vice versa*. Active PGE_2 and $PGF_{2\alpha}$ are metabolised by 15-hydroxy prostaglandin dehydrogenase (HPGD) to PGE_2 (PGEM) and $PGF_{2\alpha}$ (PGFM) metabolites. Arachidonic acid and active $PGF_{2\alpha}$ and PGE_2 are highlighted in **bold**, whereas precursors and metabolites are depicted by doted frames. Associated enzymes are shown in *italics*. Modified after (Fortier *et al.* 2008) and (Madore *et al.* 2003).

Prostaglandin E_2 and $F_{2\alpha}$ exert their role through specific membrane receptors coupled to G proteins and second messengers (Olofsson & Leung 1994, Arosh *et al.* 2004). Multiple subtypes of PGE_2 receptors (PTGER1–4) are linked to different second messenger systems (Narumiya 1997). Expression of PTGER1 and PTGER3 is either week or absent in canine CL (Kowalewski *et al.* 2008), but PTGER2 and PTGER4 are involved in PGE_2-mediated mechanisms (Kowalewski *et al.* 2009). These subtypes are coupled to adenylate cyclase increasing cAMP, leading to activation of the protein kinase A signalling pathway (Marsh & Lemaire 1974, Arosh *et al.* 2004). Null mutants of *PTGER2* exhibit ovulation and peri-implantation problems (Kennedy *et al.* 1999, Tilley *et al.* 1999). The receptor for $PGF_{2\alpha}$ belongs to the seven-transmembrane family of G protein-coupled receptors (PTGFR), generating two second messengers (Sugimoto *et al.* 1994). Mice with knockout of *PTGFR* do not undergo luteal regression at the end of pregnancy and fail to give birth (Sugimoto *et al.* 1997).

Contribution of steroids and prostaglandins to the reproductive cycle

Luteal function in felid species can potentially be assessed *via* factors that are discussed as luteotrophic in other mammalian species (Niswender *et al.* 2000). In the bovine CL, P4 suppresses apoptotic cell death and prevents structural regression by down regulation of apoptotic factors (Rueda *et al.* 2000). It further maintains luteal function by stimulating P4 synthesis (Rothchild 1981). Treatment of bovine luteal cells with P4 decreases $PGF_{2\alpha}$ production (Pate 1988). Oestrogen sustains maintenance and function of the CL in rabbits, rats and pigs (Keyes *et al.* 1979, Niswender *et al.* 2000). It enhances cholesterol supply by stimulating cholesterol synthesis, uptake and mobilisation (Stocco *et al.* 2007) and reduces secretion of uterine $PGF_{2\alpha}$ in the pig (Ford 1982). Removal of E2 causes cessation of P4 secretion in rabbits (Holt 1989). Androgens modulate the function and lifespan of the primate CL (Hild-Petito *et al.* 1991) by being direct substrate for oestrogen synthesis (Stocco *et al.* 2007). Testosterone was shown to enhance the CL lifespan of mice and rats by directly stimulating P4 production (Thordarson *et al.* 1997, Takiguchi *et al.* 2000). Prostaglandin E_2 stimulates luteal P4 secretion in humans (Marsh & Lemaire 1974), rabbits (Boiti *et al.* 2001), cattle (Kotwica *et al.* 2003) and dogs (Kowalewski *et al.* 2013). In bovine luteal cells, PGE_2 suppresses apoptosis (Bowolaksono *et al.* 2008) and as a vasodilator, it counteracts luteolytic effects of $PGF_{2\alpha}$ in ewes (Pratt *et al.* 1977, Magness *et al.* 1981).

Based on its conserved luteolytic role (McCracken *et al.* 1999, Arosh *et al.* 2004), luteal and uteroplacental $PGF_{2\alpha}$ might be involved in luteal regression in felids as well. Prostaglandin $F_{2\alpha}$ decreases luteal synthesis of P4 in cows, ewes, humans and sows (Niswender *et al.* 1994). It affects P4 production by interfering with cholesterol transport in steroidogenic cells (Mclean *et al.* 1995) or by enhancing P4 metabolism (Stocco *et al.* 2000). The functional luteal regression is initiated by $PGF_{2\alpha}$ secreted from the uterus in most non-primate mammalian species, *e.g.*, in pig (Patek & Watson 1983), cattle (Kobayashi *et al.* 2002) and horse (Watson & Sertich 1990). The release of uterine $PGF_{2\alpha}$ triggers luteal production of $PGF_{2\alpha}$ (Tsai & Wiltbank 1997, Arosh *et al.* 2004), which might be crucial for completion of structural luteal regression (Diaz *et al.* 2002, Hayashi *et al.* 2003). In primates, luteal regression is not mediated by uterine $PGF_{2\alpha}$ and $PGF_{2\alpha}$ appears to be of luteal origin (Nagle *et al.* 2005). During prepartal luteolysis in dogs, luteolytic $PGF_{2\alpha}$ originates from the fetoplacental complex (Luz *et al.* 2006, Kowalewski *et al.* 2010).

Aims

The aim of the dissertation is to comparatively characterise selected steroid and PG biosynthesis pathways in CL of the domestic cat and the two European lynx species to deduce their regulatory contribution to the luteal lifespan. Potential luteotrophic and luteolytic factors should be identified in relation to the different feline reproductive strategies. In cats, the CL of non-pregnant luteal phases are included for comparison to pregnancy, during which CL are likely influenced by the fetoplacental complex. In lynxes, perCL of prooestrus (pre-mating) are analysed in comparison to metoestrus (post-mating), when perCL might also be under the influence of freshCL. In a supplementary study, the luteolytic capacity of the feline placenta to influence prepartal luteal regression in cats is determined. Feline-specific gene sequences are identified for gene expression studies. Expression of selected proteins is investigated using commercial and canine-specific antibodies. Commercial and in-house assays are applied for intraluteal and plasma hormone measurements. The research is conducted within the *Lynx Research Project* of the IZW, in which further regulatory mechanisms of the CL function, *e.g.*, receptivity to steroids and expression of apoptosis-related factors, and the histomorphology of feline CL are analysed. The comprehensive research should contribute to understand the unique reproductive strategy of lynxes.

Summary of the research articles

The following shows a summary of each article included in the dissertation with a specification of the author contributions. All authors declared that there is no conflict of interest.

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Article 1: Zschockelt *et al.* 2014 J Steroid Biochem Mol Biol

Corpora lutea of pregnant and pseudopregnant domestic cats reveal similar steroidogenic capacities during the luteal life span

Zschockelt L [1], Amelkina O [1], Siemieniuch MJ [2], Koster S [1], Jewgenow K [1] and Braun BC [1]

The aim of the study was to present the first comprehensive analysis of conventional and alternate intraluteal steroid biogenesis pathways in the domestic cat. It was assumed that the time dependent shift in onset of prepartal and cyclic luteal regression is caused by differences in steroidogenic capacities of CL from pregnant and non-pregnant luteal phases. Ovaries and uteri were collected after ovariectomy or ovariohysterectomy of domestic cats from local animal shelters and clinics (Berlin, Olsztyn). The isolated CL of pregnant (PR) and pseudopregnant (PP, non-pregnant) luteal phases were assigned to four sequential luteal stages: formation (F), development/maintenance (D/M), early regression (ER) and late regression (LR). For pregnancy this included days 2–5 (F), days 14–36 (D/M), days 38–39 (ER) and days 48–63 (LR), respectively PP1 (F), PP2 (D/M), PP3 (ER) and PP4 (LR) for pseudopregnancy. The day of pregnancy was determined by the stage of preimplantation embryos flushed from the oviduct, the diameter of the gestation chamber or the foetal crown-rump length. Because the time of ovulation was unknown and embryos or foetuses were absent, the CL stages of the non-pregnant luteal phase were determined histomorphologically. *Corpora albicantia* (CA) were not assigned to either pregnant or non-pregnant luteal phases. Cat-specific gene sequences were analysed by polymerase chain reaction (PCR) for the steroidogenic enzymes *HSD17B2*, *HSD17B7* and *SRD5A1*. The relative mRNA levels of *STAR*, *CYP11A1*, *CYP17A1*, *CYP19A1*, *HSD17B1*, *HSD17B2*, *HSD17B7*, *HSD3B1* and *SRD5A1* were investigated by quantitative PCR (qPCR). Fragments of reference genes (*B2M*, *BACT*, *GAPDH*, *GLS*, *TBP,* and *RPS7*) were amplified to calculate a multiple normalisation factor. Protein expression was analysed for HSD3B by Western Blot (WB) and the intraluteal levels of P4, oestrogens and androgens were determined by Enzyme-Linked Immunosorbent Assay (EIA).

Results of the study:

1. During the non-pregnant luteal phase, mRNA expression of *STAR*, *HSD3B1*, *CYP19A1* and *HSD17B7* decreased from formation of the CL onwards. In contrast, expression of *SRD5A1* increased with progression through stages of the non-pregnant CL. Expression of *CYP11A1* and *CYP17A1* was unaffected by the luteal stage. Only mRNA for *HSD17B2*

was not detectable in CL of the different stages. In general, mRNA expression profiles of steroidogenic enzymes during pregnancy reflected the profiles of pseudopregnancy.

2. The intraluteal HSD3B protein expression (~42 kDa) was highest in the D/M-stage of CL and declined during the subsequent luteal phases of pregnancy and pseudopregnancy.
3. The levels of intraluteal steroids decreased, whereby oestrogen and androgen levels were higher in the F-stage compared to the following luteal stages of pseudopregnancy. Concentrations of P4 were higher in the D/M-stage compared to the ER-stage and LR-stage.

Contribution of authors:

LZ drafted the article, supported sequence analyses, performed gene expression analysis, participated in EIA, conducted statistical analyses. *OA* collected, preserved and categorised the samples, participated in discussion. *MJS* collected additional samples and participated in discussion. *SK* carried out WB analysis. *KJ* responsible for project design, participated in study design and coordination. *BCB* responsible for project and study design, coordination, performed/supported sequence and WB analyses. All co-authors revised the article.

Article 2: Zschockelt *et al.* 2015 J Steroid Biochem Mol Biol

Comparative analysis of intraluteal steroidogenic enzymes emphasises the functionality of fresh and persistent *corpora lutea* during pro- and metoestrus in the lynx

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The study aimed to provide a detailed analysis of the steroidogenic capacity of fresh and perCL during prooestrus and metoestrus in the two European lynx species. It was suggested, that despite constantly high plasma P4 levels in lynxes, onset of oestrus is allowed by a temporal reduction of steroid biogenesis in perCL, compared to the period after ovulation, in which the steroidogenic capacity was proposed to be maintained in perCL. Ovarian tissue of five free-ranging Eurasian lynxes (EL1–EL5) was collected prior to mating (Feb 2011, prooestrus) from carcasses obtained after controlled, legal hunting (SNO, Norway). The CL were characterised as persistent (early regression stage). For medical and management reasons, two captive Iberian lynxes (IL1, IL2) of the ILCBP (Portugal, Spain) were ovariohysterectomised on day 7 *p.c.* (Feb 2013, metoestrus). Ovaries showed coexistence of perCL (previous oestrus cycles, maintenance stage) and freshCL (recent ovulation, formation stage). Additional captive Iberian lynxes (ILCBP) were included for blood sampling during the annual

cycle: prooestrus (Dec–Jan), metoestrus (Feb), pregnancy (Mar), and prolonged dioestrus (Apr–Nov). Analysis of lynx-specific gene sequences was performed by PCR for *CYP11A1*, *CYP17A1*, *HSD17B1*, *HSD17B2*, *HSD17B7*, *SRD5A1* and the reference genes *B2M*, *BACT*, *GAPDH*, *GLS*, *RPS7* and *TBP*. Relative amounts of mRNA copies (including *STAR*, *HSD3B1*, *CYP19A1*) were analysed by qPCR. Protein expression of CYP11A1, CYP17A1 and CYP19A1 was analysed by immunohistochemistry (IH). Protein expression of HSD3B was investigated by IH and WB. Intraluteal and plasma steroid levels were determined by EIA.

Results of the study:

1. At metoestrus, relative amounts of mRNA copies for *STAR*, *CYP11A1*, *CYP19A1*, *HSD17B7* and *SRD5A1* were higher in perCL compared to freshCL. In contrast, the *HSD3B1* mRNA amount was elevated in freshCL compared to perCL of metoestrus. For *CYP17A1* and *HSD17B1* no effect of the luteal stage was detected. The mRNA of *HSD17B2* was not detectable in CL of the different stages.
2. Strong protein signals of CYP11A1 and CYP17A1 were detected independently of the CL stage in metoestrus, but expression was less evident in prooestrous perCL. The presence of CYP19A1 protein was confirmed in each CL stage. Protein for HSD3B was substantially present in freshCL, whereas signals were absent in all perCL.
3. Expression of HSD3B coincided with high intraluteal oestrogen levels in freshCL but the enzyme pattern was less concordant with intraluteal P4 and androgen levels. Lowest intraluteal steroid levels were detected in prooestrus perCL. Serum P4 and oestrogen levels were constantly high between prooestrus and prolonged dioestrus of Iberian lynxes.

Contribution of authors:

LZ drafted the article, supported sequence analyses, performed gene expression analysis, assisted in IH, participated in EIA, conducted statistical analysis. *OA* preserved and categorised the samples, participated in discussion. *SK* carried out WB analysis and IH. *JP* collected samples from the Statens Naturoppsyn. *MWO* partly provided antibodies and assistance for IH. *RS* provided samples from the Iberian Lynx Conservation Breeding Programme. *AV* enabled blood sampling within the Iberian Lynx Conservation Breeding Programme. *KJ* responsible for project design, participated in study design and coordination. *BCB* responsible for project and study design, coordination, performed/supported sequence and WB analyses. All co-authors revised the article.

Article 3: Zschockelt *et al.* 2015 *currently under review in Reproduction*

Contribution of luteal prostaglandin synthesis and reception to lifespan of feline *corpora lutea*

Zschockelt L [1], Amelkina O [1], Siemieniuch MJ [2], Kowalewski MP [3], Dehnhard M [1], Jewgenow K [1] and Braun BC [1]

The aim was to deduce implications of intraluteal PGs to (i) initiate onset of luteal regression during the pregnant and non-pregnant luteal phases in cats, to (ii) maintain CL persistence in lynxes, and to (iii) transiently reduce the functionality of perCL prior to oestrus. The CL of domestic cats were assigned to pregnancy (preimplantation: PRI, days 2–10; postimplantation: POI, days 14–36; regression: R, days 38–48), the non-pregnant luteal phase (formation: F, development/maintenance: D/M, early regression: ER, late regression: LR) or the *corpus albicans* (CA). The CL of five Eurasian lynxes (EL1–EL5; SNO, Norway) collected at prooestrus were characterised as persistent. From two Iberian lynxes (IL1, IL2; ILCBP, Portugal, Spain) perCL and freshCL were collected at day 7 *p.c.* (metoestrus). Additional CL representing the annual cycle were obtained from legally hunted or road killed Eurasian lynxes (SVA, Sweden): prooestrus (Jan–Feb), oestrus (Mar), metoestrus (Apr), and prolonged dioestrus (Sep–Dec). Feline-specific gene sequences (*PTGS2/COX2*, *PTGES*, *PGFS/AKR1C3*, *PTGER2*, *PTGER4*, and *PTGFR*) were analysed by PCR. The relative mRNA expression (Real Time PCR Miner Method) or the relative amount of mRNA copies (standard curve) were analysed by qPCR. Protein expression of PTGES, PTGER4, PGFS/AKR1C3 and PTGFR was determined by IH. Intraluteal PGE_2 and $PGF_{2\alpha}$ levels were analysed by EIA.

Results of the study:

1. During the non-pregnant luteal phase, the relative mRNA expression of *PTGER4* was lower in the D/M-stage compared to the LR-stage. *PTGFR* expression was higher in the D/M-stage, ER-stage and LR-stage compared to the F-stage. At metoestrus, the relative amount of mRNA copies for *PTGFR* was higher in perCL compared to freshCL. The expression of *PTGS2/COX2*, *PTGER2* and *PTGER4* tended to be higher in perCL of prooestrus, compared to metoestrous CL.
2. During the pregnant and non-pregnant luteal phase of cats and between prooestrus and metoestrus of lynxes, expression of PTGES and PTGER4 protein was independent of the

luteal stage. Protein expression of PGFS/AKR1C3 was low in cats and absent in lynxes. In contrast, expression of PTGFR was evident in nearly all investigated CL.

3. Levels of PGE_2 and $PGF_{2\alpha}$ were unaffected by the luteal and reproductive stages in cats. In general, PGE_2 levels were substantially higher compared to $PGF_{2\alpha}$ levels in cats and lynxes and the PGE_2:$PGF_{2\alpha}$ ratio showed clear prevalence towards PGE_2. Although annual intraluteal levels of PGE_2 and $PGF_{2\alpha}$ were rather constant, the levels tended to be higher in perCL compared to freshCL in metoestrous lynxes.

Contribution of authors:

LZ drafted the article, performed sequence and gene expression analyses, carried out IH, participated in EIA, conducted statistical analysis. *OA* collected, preserved and categorised the samples, participated in discussion. *MJS* partly provided facilities and assistance in gene analysis. *MPK* partly provided facilities and assistance in IH. *MD* provided in-house and commercial EIAs. *KJ* responsible for project design, participated in study design and coordination. *BCB* responsible for project and study design, coordination. All co-authors revised the article.

Article 4: Siemieniuch *et al.* 2014 Mediators Inflamm

Placental origin of prostaglandin $F_{2\alpha}$ in the domestic cat

Siemieniuch MJ [2], Jursza E [2], Szóstek AZ [2], Zschockelt L [1], Boos A [3] and Kowalewski MP [3]

The study aim was to examine the capacity of the feline placenta to synthesise $PGF_{2\alpha}$ in a time dependent manner to deduce its influence on the feline CL lifespan. For pregnant cats, the uteroplacental complex was assumed to be a possible source of luteolytic $PGF_{2\alpha}$ in late pregnancy and prior to parturition. Thirty domestic, healthy cats were ovariohysterectomised and isolated placentas were assigned to four different groups: postimplantation/early pregnancy (days 18–21 *p.c.*, 2.5–3 weeks), mid pregnancy (days 28–35 *p.c.*, 4–5 weeks), late pregnancy (days 49–56 *p.c.*, 7–8 weeks) and before parturition (days 60–64 *p.c.*, 8.5–9 weeks). Blood samples were collected from the cephalic vein and plasma was obtained after blood centrifugation. Maternal plasma PGFM was analysed by direct EIA. Sections of fixed placental fragments were used to determine the cellular localisation of PTGS2/COX2 and PGFS/AKR1C3 protein throughout pregnancy by IH. The placental levels of mRNA expression were examined by qPCR (Real Time PCR Miner Method) for *PTGS2/COX2*, *PGFS/AKR1C3* and the reference gene *CYC*. The content of PGFS/AKR1C3 protein was determined by WB. Levels of extracted placental $PGF_{2\alpha}$ were measured by EIA.

Results of the study:

1. The placental *PGFS/AKR1C3* mRNA expression was upregulated at 2.5–3 weeks of pregnancy and gradually declined towards the end of pregnancy. The mRNA expression of placental *PTGS2/COX2* was only upregulated in placentas from cats close to term compared to earlier phases of pregnancy.
2. The placental expression of PGFS/AKR1C3 protein (~34–36 kDa) was upregulated at 2.5–3 weeks of pregnancy, compared to 7–8 and 8.5–9 gestational weeks. Positive signals for protein expression of PTGS2/COX2 were abundant in the ninth week of pregnancy (last week before parturition). Only weak signals were observed for placental PTGS2/COX2 expression during earlier stages of pregnancy.
3. The levels of placental $PGF_{2\alpha}$ mirrored maternal plasma PGFM levels throughout pregnancy. Both placental $PGF_{2\alpha}$ and plasma PGFM were elevated towards the last three weeks of pregnancy compared with earlier weeks.

Contribution of authors:

MJS drafted the article, conceived and designed the study, participated in molecular and hormonal analysis. *EJP* carried out WB analysis. *AZS* participated in IH. **LZ** carried out gene expression analysis. *AB* assisted in drafting the article. *MPK* participated in design of the study and interpretation of the results, assisted in drafting the article. All co-authors revised the article.

Discussion

A comparative characterisation of steroid and PG synthesis pathways in CL of domestic cats and European lynx species is presented. Luteal and placental tissue of pregnant and non-pregnant cats, and luteal tissue of free-ranging Eurasian (prooestrus, pre-mating) and captive Iberian (metoestrus, post-mating) lynxes were analysed. Additional animals were acquired for collection of luteal tissue (Eurasian lynxes) and blood sampling (Iberian lynxes) throughout the annual cycle. Feline-specific gene sequences of steroid and PG synthases, PG receptors and reference genes were identified for a detailed molecular biological analysis. Selected proteins were investigated using commercial and canine-specific antibodies. Intraluteal and serum levels of steroids and PGs were measured with in-house and commercial hormone assays. Potential luteotrophic and luteolytic factors of luteal (P4, oestrogen, PGE_2) and placental ($PGF_{2\alpha}$) origin were identified and discussed for their contribution to the feline luteal lifespan.

Relation of steroid biogenesis pathways to luteal histomorphology

During the early CL formation of most species, oestrogen secreting follicular cells transform to mainly P4 synthesising luteal cells (Slomczynska & Tabarowski 2001, Havelock *et al.* 2004). The changes in luteal steroidogenic capacities in the domestic cat (Zschockelt *et al.* 2014) and the two European lynx species (Zschockelt *et al.* 2015) are reflected by histomorphological transformations of the CL (Amelkina *et al.* 2015, Amelkina *et al.* 2015). In cats, the preimplantative CL formation, its development/maintenance and partly the early and late regression, are depicted by luteal cells with lipid vesicles and vesicular nuclei, indicating steroid-secreting activity (Magoffin 2005, Amelkina *et al.* 2015). Formation of the cat CL is accompanied by transformation of follicular theca and granulosa cells to steroidogenic luteal cells and hypertrophy, leading to a polyhedral cell form and lipid accumulation (Amelkina *et al.* 2015). There is high histomorphological similarity to freshCL of metoestrous lynxes (Amelkina *et al.* 2015). The CL formation in both felids is reflected by high intraluteal P4, oestrogen and androgen levels (Zschockelt *et al.* 2014, Zschockelt *et al.* 2015). The steroidogenic enzyme responsible for the initial cholesterol cleavage (CYP11A1; Figure 3) is constantly expressed in cat CL (Zschockelt *et al.* 2014) and is evident to different extents in fresh and perCL of prooestrous and metoestrous lynxes (Zschockelt *et al.* 2015), suggesting that *de novo* steroidogenic capacity is acquired directly after luteinisation and is sustained throughout the luteal phases. Likewise, *CYP11A1* is constitutively expressed in the rat CL following luteinisation (Goldring *et al.* 1987). In pregnant cats, elevated intraluteal P4 and oestrogen levels

(Zschockelt *et al.* 2014) mirror high plasma P4 and oestrogen concentrations of the early luteal phase (Verhage *et al.* 1976) (Figure 1). This is supported by high expression of *STAR* and HSD3B (Zschockelt *et al.* 2014). It was impossible to compare intraluteal and plasma levels of non-pregnant cats, because time of ovulation was unknown in the respective study (Zschockelt *et al.* 2014). In lynxes, elevated intraluteal P4, E2 and androgen levels in freshCL are consistent with elevated HSD3B expression (Zschockelt *et al.* 2015). A similar pattern was shown for other species. During the early luteal phase in dogs, CL formation is reflected by intense proliferative activity and vascularisation, concomitant to rapidly increasing steroidogenic capacity (STAR, HSD3B) (Hoffmann *et al.* 2004, Papa & Hoffmann 2011, Kowalewski 2012). In cattle, proliferation of luteal steroidogenic cells during CL development is also associated with abundant HSD3B expression (Yoshioka *et al.* 2013). The source of high intraluteal (Zschockelt *et al.* 2014) and plasma (Verhage *et al.* 1976) steroid levels during preimplantation in cats might be transforming follicular or newly formed luteal cells (Wildt *et al.* 1981). Similarly, follicle-derived E2 levels reach peak values during the preovulatory phase in dogs, but rapidly drop following ovulation (Papa & Hoffmann 2011).

The full secretory capacity of CL is achieved, when the formation is completed (Stouffer 2006). The chronic regulation of steroid biogenesis occurs *via* CYP11A1, whereas acute regulation, *i.e.*, access of cholesterol to CYP11A1, is mediated by STAR (Miller 2008) (Figure 3). During its development/maintenance, the CL of cats is progressively infiltrated by blood vessels and reaches its maximum size (Dawson 1941, Amelkina *et al.* 2015). A similar histology is seen in perCL of metoestrous lynxes, revealing signs of CL maintenance (Amelkina *et al.* 2015). In cats, a shift from high intraluteal oestrogen and androgen to high P4 synthesis, supported by high *STAR* and HSD3B expression (Zschockelt *et al.* 2014), is reflected by the transition from CL formation towards its development/maintenance (Amelkina *et al.* 2015). In pregnant cats, this histomorphological transition is consistent with increasing plasma P4 and decreasing plasma oestrogen levels (Verhage *et al.* 1976) (Figure 1). Similarly, expression patterns of luteal canine STAR and HSD3B during the luteal phase parallels the peripheral P4 content in pregnant and non-pregnant dogs (Kowalewski *et al.* 2006, Kowalewski & Hoffmann 2008, Kowalewski *et al.* 2009). However, *de novo* steroid biogenesis does not seem to be regulated *via* provision and transfer of cholesterol (STAR) in cats, but by terminal steroidogenic enzymes (HSD3B) (Zschockelt *et al.* 2014). Likewise, synthesis of active steroids in lynxes seems to be regulated *via* HSD3B, rather than STAR (Zschockelt *et al.* 2015). Synthesis of all steroids is reduced in perCL of metoestrous lynxes, concomitant to reduced expression of HSD3B, but contrary to high *STAR* expression (Zschockelt *et al.* 2015). This is

remarkable, because in dogs synthesis of P4 depends on cholesterol transfer through the mitochondrial membranes mediated by STAR (Kowalewski & Hoffmann 2008); the progressive decrease in HSD3B expression was considered secondary (Kowalewski *et al.* 2006). Similarly, in ovine luteal cells CYP11A1 and HSD3B activity is present in excess amounts and is not the rate-limiting step in luteal P4 production (Wiltbank *et al.* 1993). Accordingly, neither the luteal amount of bovine *CYP11A1* and *HSD3B*, nor the enzyme activity is affected by $PGF_{2\alpha}$ treatment (Rodgers *et al.* 1995), whereas treatment of ewes or cows with $PGF_{2\alpha}$ dramatically reduces concentrations of *STAR* (Wiltbank *et al.* 1993, Pescador *et al.* 1996).

It is generally accepted that loss of luteal P4 secretion precedes structural luteal regression (McCracken *et al.* 1999). During the transition from the CL development/maintenance towards regression in cats, the degree of lipid vacuolation, resembling cholesterol storage, decreases until autophagocytic vacuoles no longer react to lipid staining (Amelkina *et al.* 2015). The ongoing luteal phase is reflected by an increase of non-steroidogenic cells in expense of luteal cells (Amelkina *et al.* 2015). The early luteal regression in pregnant cats coincides with decreases in intraluteal (Zschockelt *et al.* 2014) and plasma (Verhage *et al.* 1976) levels of P4 and oestrogens (Figure 1). Accordingly, the expression of HSD3B is declined (Zschockelt *et al.* 2014). Persistent CL of prooestrus lynxes show histological signs of early regression as well (Zschockelt *et al.* 2015) and distinctly lower intraluteal steroid levels compared to metoestrous freshCL (Zschockelt *et al.* 2015). The slighter difference in luteal steroidogenic capacity between prooestrous and metoestrous perCL is not due to HSD3B, which expression is absent in all perCL. But as demonstrated by reduced expression of CYP11A1 and CYP17A1, reflected especially in decreased oestrogen levels, initial cholesterol cleavage and conversion of steroid intermediates to active steroids is limited in perCL of prooestrus. In the mid-luteal phase of dogs, luteal regression and fatty degeneration set in (Hoffmann *et al.* 2004). Similar to cats this is reflected in the gradual decline of peripheral P4 levels, concomitant to the decreased proliferative activity (Kowalewski 2012). Related to aging of the canine CL, expression of STAR and HSD3B slowly decreases (Kowalewski & Hoffmann 2008, Kowalewski *et al.* 2009). Similarly, regression of the bovine, ovine and human CL is associated with a loss in STAR expression (Juengel *et al.* 1995, Pescador *et al.* 1996, Devoto *et al.* 2001). In *corpora albicantia*, no luteal cells with vesicular nuclei are found in the cat (Amelkina *et al.* 2015) and steroid levels become basal (Verhage *et al.* 1976, Zschockelt *et al.* 2014) (Figure 1), indicating decreased steroidogenic activity. In the late bovine pregnancy, the secretory activity of CL is markedly diminished as well, due to a decline in the number of viable luteal cells and decrease of their secretory activity (Shemesh 1990).

Peculiarities of prostaglandin synthesis in *corpora lutea* of felids

In most mammals, the luteal capacity to produce PGs is induced by specific events during the luteal phase (Olofsson & Leung 1994). However, the CL of cats and lynxes exhibit a steady-state *de novo* synthetic capacity (*PTGS2/COX2*; Figure 4), independent on luteal formation, maintenance or regression (Zschockelt *et al.* 2015). In contrast, luteal growth in the early luteal phase of dogs is associated with increasing expression of *PTGS2/COX2* (Kowalewski *et al.* 2006, Kowalewski *et al.* 2009). In the rhesus monkey, luteal concentrations of PTGS2/COX2 are highest in the early luteal phase as well (Bogan *et al.* 2008). In contrast, the content of PTGS2/COX2 is high in the mouse (Sander *et al.* 2008) and rat ovary (Olofsson *et al.* 1991) during CL regression and the luteal PTGS2/COX2 activity increases from the early to the late luteal phase in humans (Mitchell *et al.* 1991). Moreover, during luteal regression there seems to be a positive auto-amplification loop, in which $PGF_{2\alpha}$ acutely activates $cPLA_2$ and induces expression of PTGS2/COX2 in the ovine CL (Rexroad & Guthrie 1979).

The selective production of PGs is generally in favour of PGE_2 rather than $PGF_{2\alpha}$ during luteal development in different species (Vijayakumar & Walters 1987, Olofsson *et al.* 1992). For example in bovines, the conversion of PGH_2 to PGE_2 by PTGES is 150-fold higher than conversion of PGH_2 to $PGF_{2\alpha}$ by PGFS (Madore *et al.* 2003). In feline CL, PG biosynthesis is preferentially directed towards PGE_2 as well (Zschockelt *et al.* 2015). However, the high ratio of PGE_2:$PGF_{2\alpha}$ synthesis is not clearly shifted between luteal stages in cats and lynxes, although a tendency for higher PGE_2 synthesis during the CL formation might be assumed for cats (Zschockelt *et al.* 2015). In lynxes this is further mirrored by high annual intraluteal PGE_2 levels (Jewgenow *et al.* 2012, Zschockelt *et al.* 2015). The constant intraluteal PGE_2 synthesis in felids neither reflects the functional and structural demise of cat CL at the end of the luteal phases (Zschockelt *et al.* 2014), nor the temporarily limited steroidogenic capacity of prooestrous perCL in lynxes (Zschockelt *et al.* 2015). In contrast, relative to $PGF_{2\alpha}$, the PGE_2 synthesis is higher in growing, lower in regressing and equal in mature bovine CL (Arosh *et al.* 2004). Similarly, PGE_2 synthesis is particularly evident during the phase of luteal formation in dogs, likely being involved in the CL development (Kowalewski *et al.* 2015).

In general, luteal synthesis of PGs is shifted towards luteolytic $PGF_{2\alpha}$ in the late luteal phase (Wiltbank & Ottobre 2003). Interestingly, CL of cat and lynxes exhibit only limited capacities to synthesise $PGF_{2\alpha}$ (Zschockelt *et al.* 2015). The constantly low intraluteal PGFS/AKR1C3 expression and low $PGF_{2\alpha}$ levels neither reflect the luteal regression at the end of the luteal phases in cats, nor early signs of CL regression in prooestrous lynxes (Zschockelt *et al.* 2015).

The canine CL is devoid of PGFS/AKR1C3 expression as well (Kowalewski *et al.* 2008). In contrast, luteal $PGF_{2\alpha}$ levels increase at the expense of PGE_2 during luteal regression in mice (Sander *et al.* 2008). Similarly, $PGF_{2\alpha}$ production in pigs rises from the mid to the late luteal phase (Patek & Watson 1976). However, the capacity of feline CL to respond to $PGF_{2\alpha}$ is already developed after the CL formation and is maintained throughout the lifespan (Zschockelt *et al.* 2015), implying that luteal regression is triggered by extraluteal $PGF_{2\alpha}$. Also in dogs, the CL rather constantly expresses *PTGFR* already following formation (Kowalewski *et al.* 2008). In contrast, *PTGFR* expression increases during the late luteal phase in bovine and pig CL, when CL become sensitive to $PGF_{2\alpha}$ (Boonyaprakob *et al.* 2003, Arosh *et al.* 2004). Likewise, during structural luteal regression in pseudopregnant mice, onset of apoptosis correlates with maximum expression of *PTGFR* (Hasumoto *et al.* 1997).

Contribution of intraluteal factors to function of *corpora lutea*

An autocrine/paracrine luteotrophic effect of P4 and PGE_2 on the CL formation and maintenance is proposed for felids (Amelkina *et al.* 2015, Zschockelt *et al.* 2015). During the luteal phase, the CL of cats is capable to express nuclear (PGR) and membrane (PGRMC1, PGRMC2) receptors of P4 (Amelkina *et al.* 2015), as well as PTGER2 and PTGER4 for binding PGE_2 (Zschockelt *et al.* 2015). This suggests that the cat CL is potentially receptive to P4 and PGE_2 throughout the lifespan. A luteotrophic role of P4 during the CL formation was shown for primates (Stouffer 2003) and dogs as well (Hoffmann *et al.* 2004, Papa & Hoffmann 2011). Moreover, activity of luteal PGE_2 seems to be responsible for CL maintenance and function during the first third of the canine CL lifespan (Kowalewski *et al.* 2008, Kowalewski *et al.* 2013). A luteotrophic role of both hormones would be in accordance with the resistance of early cat CL to $PGF_{2\alpha}$ given on days 4 and 5 or 12 and 13 *p.c.*, which has no effect on circulating P4 levels and the CL size (Wildt *et al.* 1979). Also high doses and repeated $PGF_{2\alpha}$ treatments on days 11–15 *p.c.* have almost negligible effects and luteal function recovers rapidly (Shille *et al.* 1979). On days 21–25 *p.c.*, luteal function is transiently depressed, but $PGF_{2\alpha}$ treatment does not alter the length of the luteal phase (Shille *et al.* 1979).

It must be considered, that the subcutaneous administration of $PGF_{2\alpha}$ in the above mentioned studies might have led to systemic metabolism of $PGF_{2\alpha}$ (Wheeler *et al.* 1988) and that the insensitivity of early cat CL to $PGF_{2\alpha}$ might be caused by insufficient expression of PTGFR (Zschockelt *et al.* 2015). Refractoriness to exogenous $PGF_{2\alpha}$ also occurs in the early luteal phase of pigs (Moeljono *et al.* 1976) and cows (Rao *et al.* 1979), due to low density of PTGFR in early to mid-luteal phases (Gadsby *et al.* 1990). However, it cannot be ruled out

that high intraluteal P4 and PGE_2 levels (Zschockelt *et al.* 2014, Zschockelt *et al.* 2015) sufficiently prevent regression of the early cat CL. Interestingly, Shille *et al.* even revealed an enhancement of the luteal activity by $PGF_{2\alpha}$ treatment on days 11–15 *p.c.* (Shille *et al.* 1979), suggesting that $PGF_{2\alpha}$ might exert luteotrophic effects, if PTGFR is adequately expressed, in some situations. Indeed, $PGF_{2\alpha}$ can increase P4 production in cultured bovine luteal steroidogenic cells (Okuda *et al.* 1998). Furthermore, in pseudopregnant pigs $PGF_{2\alpha}$ induces luteal production of PGE_2 (Diaz *et al.* 2000). Prostaglandin $F_{2\alpha}$ downregulates mRNA encoding *PTGS/COX* in bovine CL early in the oestrus cycle, when $PGF_{2\alpha}$ cannot cause luteal regression, which suggests a luteotrophic role of $PGF_{2\alpha}$ in this period (Tsai & Wiltbank 1998).

Compared to the non-pregnant luteal phase, the CL of pregnant cats might be additionally influenced by pregnancy-specific factors, which could prolong the CL maintenance beyond its inherent lifespan. For example, pregnancy in cats can be diagnosed and monitored by determining relaxin concentrations in urine during mid-pregnancy (de Haas van Dorsser *et al.* 2006). Relaxin was confirmed in placental tissues and in lower levels in CL, foetus, and uterus of cats (Addiego *et al.* 1987). An influence of relaxin on the CL of pregnancy can also be assumed for the Iberian lynx, in which placenta derived relaxin measured in urine and serum during the second half of pregnancy is a precise indicator of pregnancy as well (Braun *et al.* 2009). The CL of lactating cats might be influenced by luteotrophic effects of prolactin secreted from the anterior pituitary. Prolactin is elevated during the last third of pregnancy and remains elevated during the lactation period (Banks *et al.* 1983, Verstegen *et al.* 1993). This coincides with the *postpartum* presence of the cat CL (Dawson 1946, Amelkina *et al.* 2015). Beside placental or pituitary factors, additional sources might act on the feline CL lifespan. In sheep and rats, PGE_2 produced by the blastocyst, or secreted by the endometrium, acts as a luteotrophic factor that supports initial steps of pregnancy (Magness *et al.* 1981, Kennedy 1983). In pigs, oestrogen production by the implanting blastocyst diverts secretion of endometrial $PGF_{2\alpha}$ leading to absence of $PGF_{2\alpha}$ in uterine venous blood (Gross *et al.* 1988). The equine conceptus alters the ratio of PGE_2:$PGF_{2\alpha}$ in the uterine vein and PGE_2 stimulates continued luteal function during pregnancy (Niswender *et al.* 2000). Also the foetus of ruminants prevents uterine secretion of $PGF_{2\alpha}$ (Wiltbank *et al.* 1992).

The refractoriness of lynx CL to structural regression might be caused by auto-amplification of intraluteal steroids and PGE_2, attenuating luteolytic and apoptotic processes. Progesterone and PGE_2 synthesis is sustained in perCL (Zschockelt *et al.* 2015, Zschockelt *et al.* 2015), concomitant to high expression of respective receptors (Amelkina *et al.* 2015, Zschockelt *et*

al. 2015). The nuclear (ESR1, ESR2) and membrane receptors (GPER1) of oestrogen in fresh and perCL are reversely expressed, but in tendency higher expression is evident in perCL (ESR1 and GPER1) (Amelkina *et al.* 2015). In Iberian lynxes, perCL differ from freshCL in higher expression of the androgen receptor (AR) as well (Amelkina *et al.* 2015). In this regard it was suggested that freshCL might be an additional source of luteotrophic steroids (P4, oestrogens and androgens) to highly receptive perCL, and that the functional and structural stage of perCL changes under the influence of a new ovulation, returning from early regression to CL maintenance (Amelkina *et al.* 2015). This is likely, as progestin priming increases ovarian sensitivity to gonadotrophin stimulation and improves luteal function in the cat (Stewart *et al.* 2012). In humans (Baird *et al.* 2003), baboons (Castracane *et al.* 1998) and rats (Goyeneche *et al.* 2003), the CL can be rescued by gonadotrophic support as well, accompanied by increased P4 concentrations. A *postpartum* reactivation of luteal function occurs after a transient *prepartum* luteal regression in the woodchuck (Concannon *et al.* 1997). In this respect, the morphology of cat CL during lactation resembles the appearance of CL during the second week of pregnancy (Amelkina *et al.* 2015). This most likely is influenced by prolactin, making it a potential luteotrophic factor, which might influence CL persistence in lynxes as well.

On the other hand, it was hypothesised that perCL might supplement P4 secretion of freshCL to compensate luteal insufficiencies and sustain pregnancy in lynxes (Woshner *et al.* 2001, Jewgenow *et al.* 2014). This was based on the observation that incidence of resorption or early abortion is especially high within primiparous lynxes (Jewgenow *et al.* 2014). In equids, accessory CL are formed, responsible for continued secretion of P4 required for maintaining pregnancy, until a shift towards placental hormone production occurs (Niswender *et al.* 2000). Similarly, multiple accessory CL of elephants support the extremely long pregnancy as an additional source of P4 (Hildebrandt *et al.* 2011). Although the amount of P4 synthesised by freshCL markedly exceeds this of perCL (Carnaby *et al.* 2012, Zschockelt *et al.* 2015), these levels appear insufficient for pregnancy maintenance in some cases, making a P4 support by perCL more likely. Moreover, a luteotrophic effect of elevated PGE_2 synthesis by perCL might positively influence P4 secretion by freshCL (Zschockelt *et al.* 2015).

The findings on substantial annual intraluteal P4 synthesis in Eurasian lynxes (Carnaby *et al.* 2012) strengthen the hypothesis that perCL directly support constantly elevated plasma P4 levels (Göritz *et al.* 2009, Painer *et al.* 2014) (Figure 2). This was assumed to prevent folliculogenesis and ovulation outside the breeding season by a negative feedback of P4 on gonadotrophic secretion (Göritz *et al.* 2009, Painer *et al.* 2014). Thus, lynxes enter a prolonged dio-

estrus, in contrast to the anoestrus period of other felids, ensuring a strict seasonal monooestrous cycle (Painer *et al.* 2014). In cows, the prolonged luteal phase, including maintenance of plasma P4 levels due to CL persistence, is an inhibitor of reproductive performance as well, preventing normal oestrus cyclicity (Magata *et al.* 2012). Similarly, P4 suppresses growth of developing ovarian follicles in rabbits, rodents and non-human primates (Peluso 2006). In dogs, the non-seasonal monooestrus seems to be partly ensured by the possibility to prolong the CL lifespan in non-pregnant dogs, which can exceed that in pregnant animals (90 *versus* 60 days) (Concannon 1993, Nohr *et al.* 1993). According to elevated prolactin levels during the lactation period of cats, in which no signs of oestrus are shown until after weaning in many cases (Banks *et al.* 1983) and concomitant with presence of CL at least until day 63 after parturition (Dawson 1946, Amelkina *et al.* 2015), CL persistence and thus the monooestrus of lynxes might further be ensured by a potential luteotrophic role of prolactin.

A reproductive advantage of constant P4 levels in lynxes might be that endogenous P4 exposure exerts a priming influence on ovarian quiescence that might sensitise the ovary to be highly responsive to FSH and LH stimuli prior to the next breeding season (Pelican *et al.* 2010). This ovarian suppression prior to gonadotrophin stimulation improves ovarian follicular response to gonadotrophin stimulus in cows (Bo *et al.* 2002), sheep (Gonzalez-Bulnes *et al.* 2005) and humans (Al-Inany 2002) as well. Lynxes apparently evolved special adaptations to maintain CL persistence. These might enhance survival of offspring, because birth falls into moderate climate conditions, with high prey abundance (Palomares 2001). Moreover, male Eurasian lynxes express seasonal sperm production matching the breeding period of females (Göritz *et al.* 2006, Jewgenow *et al.* 2006).

Regulation of *corpus luteum* regression by luteal and placental factors

In non-pregnant cats, any pregnancy-specific luteolytic signal is missing. It was postulated, that in the endometrium of non-pregnant cats an E2-stimulated $PGF_{2\alpha}$ output, primed by a period of P4 dominance, is involved in cyclic luteal regression (Siemieniuch *et al.* 2010). The feline endometrium is capable of synthesising $PGF_{2\alpha}$ (Siemieniuch *et al.* 2010, Siemieniuch *et al.* 2013) and the vascular uteroovarian plexus could facilitate transport of luteolytic $PGF_{2\alpha}$ from the uterus to the ovaries (Delcampo & Ginther 1974, Jewgenow *et al.* 2012). However, ovarian function in non-pregnant cats is neither affected by hysterectomy, nor by diversion of the uterine venous blood from the ovarian vasculature, suggesting that endometrial $PGF_{2\alpha}$ has no local or systemic influence on the duration of the CL lifespan (Wheeler *et al.* 1988). Moreover, prolonged administration of large doses of $PGF_{2\alpha}$ fails to induce luteal regression and

leads to severe side effects (Shille *et al.* 1979), suggesting that this treatment does not resemble physiological cyclic regression. In non-pregnant felids neither intraluteal (Zschockelt *et al.* 2015), faecal and urinary (Finkenwirth *et al.* 2010, Dehnhard *et al.* 2012), nor plasma (Painer *et al.* 2014) (Figure 2) levels show changes in $PGF_{2\alpha}$ or PGFM profiles, suggesting that cyclic luteal regression is not actively regulated by luteal or endometrial $PGF_{2\alpha}$. In dogs, the luteal regression during pseudopregnancy is a rather permissive process (Kowalewski 2012, Kowalewski 2014) without extraluteal luteotrophic stimuli and involvement of uterine or internal $PGF_{2\alpha}$ as well (Okkens *et al.* 1985, Hoffmann *et al.* 2004). Also in primates, luteal regression is not mediated by uterine $PGF_{2\alpha}$ (Stouffer 2006). This is in contrast to non-pregnant rats (Pharriss & Wyngarden 1969), ewes (McCracken *et al.* 1970) or mares (Ginther *et al.* 1972) in which luteal regression is triggered by endometrial $PGF_{2\alpha}$.

However, in cats the cyclic regression is not as prolonged as in the non-pregnant dog (Paape *et al.* 1975). In dogs, the CL of pseudopregnancy is sufficient to support a luteal phase that completes its inherent lifespan by undergoing an extended period of luteal regression compared to pregnancy (Kowalewski 2014). This occurs although support or supplementation by any pregnancy-specific mechanism is absent, as no placental P4 production or placental gonadotrophin is present (Concannon 2009). The shortened luteal phase in non-pregnant cats might either be caused by the absence of external luteotrophic factors, which prolong the CL lifespan during pregnancy, or there exists an inherent regression programme independent of $PGF_{2\alpha}$. A combination of both might be considered as well. Whatever the mechanism of the relatively fast luteal regression in non-pregnant cats might be, it seems to be reproductive advantageous compared to the monooestrous dog, in which acute internal regression mechanisms are absent. Because development of CL prevents another ovarian cycle (Wildt *et al.* 1981), the evolution of luteal regression in non-pregnant cats already after 25–40 days *p.c.* (Figure 1) might increase the reproductive potential of this induced ovulating species, because the opportunity for conception is given after a relatively short interval of time.

In cats, contradictory to preimplantative intraluteal (Zschockelt *et al.* 2014) and plasma E2 (Verhage *et al.* 1976) elevations and decreasing oestrogenic capacity of the CL (Zschockelt *et al.* 2014), receptiveness towards oestrogens increases with progression of the luteal phase (Amelkina *et al.* 2015). Serum E2 levels slightly increase with a surge prior to parturition, when P4 concentrations are low (Verhage *et al.* 1976) (Figure 1), indicating that oestrogen might play a role in luteal regression. This is similar to cattle, sheep, goat and pig in which a pregnancy-specific E2 increase is shown (Hoffmann 1990). In cows, oestrogen increases uter-

ine $PGF_{2\alpha}$ secretion to reinforce uterine contractility prior parturition (Fuchs *et al.* 1997) and it promotes functional luteolysis by redistributing $PGF_{2\alpha}$ receptors in primate luteal cells (Kim *et al.* 2015). Also E2 from the developing preovulatory follicle triggers the release of uterine $PGF_{2\alpha}$ in sheep (Fairclough *et al.* 1980). Related to this, only during the late pregnancy $PGF_{2\alpha}$ seems to play a physiological luteolytic role in the cat, *i.e.*, intraluteal (*e.g.* PGE_2) and extraluteal (*e.g.* relaxin and prolactin) luteotrophic support of CL maintenance might be actively curtailed prior parturition by placental $PGF_{2\alpha}$ (Siemieniuch *et al.* 2014).

In cats, placental PTGS2/COX2 is upregulated at the end of pregnancy, concomitant to elevated levels of placental $PGF_{2\alpha}$ and plasma PGFM (Siemieniuch *et al.* 2014). Similarly, expression of PTGS2/COX2 is elevated in foetal trophoblast cells in prepartal dogs and the increase in placental $PGF_{2\alpha}$ synthesis (Kowalewski *et al.* 2010) is linked to increased levels of plasma PGFM (Nohr *et al.* 1993, Hoffmann *et al.* 1994). In previous studies, feline PGFM profiles were measured non-invasively in urine and faeces, whereby PGFM levels increased during the last trimester of pregnancy with a prepartal peak (Finkenwirth *et al.* 2010, Dehnhard *et al.* 2012). The prepartal $PGF_{2\alpha}$ release is observed in all domestic animal species investigated so far and is associated with induction of labour by $PGF_{2\alpha}$ effects on cervical softening, uterine contractile activity and constriction of uterine blood vessels (Hoffmann *et al.* 1994). The concomitant fall in serum P4 is prerequisite for parturition, *e.g.*, *via* disinhibition of myometrical activity (Concannon 2009). However, placental $PGF_{2\alpha}$ release is further directly related to induction of luteal regression in cows and sheep (Fairclough *et al.* 1975, Lewis & Warren 1977). In cats, this seems to be the case too, because elevated peripheral PGFM concentrations during the last trimester coincide with intraluteal and plasma P4 decreases (Verhage *et al.* 1976, Zschockelt *et al.* 2014) and histological signs of early luteal regression on days 38–39 *p.c.* (Amelkina *et al.* 2015). The reduced P4 plasma levels, concomitant to slight plasma E2 elevations in prepartal cats (Figure 1), might coincide with folliculogenesis and thus with onset of a new cycle (Wildt *et al.* 1981).

The rapid *prepartum* increase of plasma PGFM in dogs is reflected by a sharp decrease of P4 only 12–24 h prior to parturition, sufficient for onset of labour (Nohr *et al.* 1993, Concannon 2009). In cats, prepartal luteal regression needs to occur in spite of constant luteotrophic support by intraluteal PGE_2 (Zschockelt *et al.* 2015). This might explain, why the decrease of plasma P4, already beginning after days 25–30 *p.c.* (Verhage *et al.* 1976), is rather gradual (Figure 1). That the luteotrophic action can be overcome by active luteolytic $PGF_{2\alpha}$ mechanisms is likely, as the intraluteal infusion of PGE_2 can inhibit $PGF_{2\alpha}$-induced luteal regression

in monkeys, but a similar treatment during late luteal regression is no longer sufficient to extend the CL life span (Zelinski-Wooten & Stouffer 1990). Interestingly, in pregnant cats PTGFR expression is evident from the early CL phase onwards (Zschockelt *et al.* 2015), although the placental $PGF_{2\alpha}$ release occurs during later stages of pregnancy (Siemieniuch *et al.* 2014). This pattern might have been evolved to curtail any potential side effects of elevated endogenous $PGF_{2\alpha}$ levels during the whole third trimester, which are not related to luteal regression. It would further explain, why $PGF_{2\alpha}$ administered on fully developed CL after day 33 (Verstegen *et al.* 1993) or day 40 of gestation (Nachreiner & Marple 1974) can exert a luteolytic effect and induce abortion in cats. A similar mechanism of highly efficient capturing of potent uterine $PGF_{2\alpha}$ to minimise side effects, next to a short half-life in peripheral circulation, was suggested for the CL in mares (Ginther 2012).

The reduction of luteal blood flow by $PGF_{2\alpha}$ initiates luteal regression by depriving substrates for steroid biogenesis and luteotrophic support in several species (Pharriss *et al.* 1970, Nett *et al.* 1976). However, $PGF_{2\alpha}$ of luteal origin is not responsible for early luteal regression in prooestrous lynxes (Zschockelt *et al.* 2015) and onset of oestrus is not reflected in urinary and faecal PGFM profiles (Finkenwirth *et al.* 2010, Dehnhard *et al.* 2012). Moreover, despite artificially induced regression with a $PGF_{2\alpha}$ analogue, perCL are preserved in structure for at least two subsequent years and blood supply and steroid production reconstitute (Painer *et al.* 2014, Painer *et al.* 2014). Interestingly, also perCL of metoestrus retain their functional and structural integrity irrespectively of high $PGF_{2\alpha}$ receptiveness (Zschockelt *et al.* 2015, Zschockelt *et al.* 2015). In contrast, in cows acute changes in modulation of vascular stability by $PGF_{2\alpha}$ are accompanied by apoptotic signalling cascades culminating at the end of functional regression or during early structural regression (Berisha *et al.* 2010). Expression of apoptosis-related factors, such as CASP3 and BCL2, is present in all investigated CL stages of cat and lynxes (Amelkina *et al.* 2015). Expression of the active CASP3 protein in cats might implement the apoptotic signal leading to final structural regression during the late luteal phase (Amelkina *et al.* 2015). Similarly in dogs, CASP3 seems to be involved in functional and structural regression (Luz *et al.* 2006). The expression of the pro-survival factor BCL2 is high in perCL of lynxes and it was hypothesised that action of BCL2 might inhibit transmission of any luteolytic signal *via* the intrinsic apoptotic cascade (Amelkina *et al.* 2015). Thus, although perCL in lynxes express apoptosis-related factors, the final structural CL demise is prevented and structural integrity is prolonged, suggesting that any inherent regression programme, if existent, is not executed as it is proposed for the cat.

In lynxes, signs of early regression at prooestrus (Amelkina *et al.* 2015), in contrast to elevated annual plasma and intraluteal P4 levels (Carnaby *et al.* 2012, Painer *et al.* 2014) (Figure 2), suggest that a potential seasonal luteotrophic support is temporarily ceased. The photoperiod is responsible for the regulation of reproductive seasonality in different species (Ortavant *et al.* 1985, Freeman *et al.* 2000). And also felids show a broad variety of reproductive cyclicity, mostly dependent on photoperiod (Brown 2011). It is suggested that the photoperiod response system acts on secretion of prolactin *via* melatonin, as shown for several seasonal breeders (Dupre 2011). For example in ewes, the effect of the photoperiod on reproductive activity is mediated by melatonin secretion from the pineal gland (Daveau *et al.* 1994). In this regard, oestrus in cats is reversibly suppressed with melatonin implants (Gimenez *et al.* 2009) by mimicking shortened day light periods and suppressing ovarian follicle development (Leyva *et al.* 1989). In contrast, folliculogenesis in cats is stimulated by exposure to continuous light (Leyva *et al.* 1989). Similarly, the reproductive seasonality in lynxes might exhibit reproductive responses to photoperiodic changes. It might be hypothesised that the prolonged dioestrus (including autumn and winter) occurs during decreased photoperiod, in which elevated melatonin levels might inhibit ovarian activity. Withdrawal of the prolonged melatonin inhibition through lengthening of the photoperiod before the breeding season (beginning of the year) might be associated with temporary luteal regression in lynxes. Thus, the reduction in local steroid synthesis in prooestrous lynxes (Zschockelt *et al.* 2015) would allow folliculogenesis and subsequent onset of oestrus despite elevated plasma P4 levels, as suggested earlier (Göritz *et al.* 2009, Painer *et al.* 2014).

Conclusion

The feline CL is a major site of steroid and PGE_2 production, and target for steroids and PGs. *Corpora lutea* of cat and lynx exhibit similar capacities to synthesise steroids and PGs. The steroidogenic capacity is associated with different luteal and reproductive stages in cats, being highest during CL formation and the development/maintenance. During the luteal lifespan of pregnant and non-pregnant cats, CL of same histomorphological stages show similar steroidogenic capacities. The functional demise of CL equally mirrors the ongoing loss of *de novo* steroid biogenesis. In perCL of lynxes, *de novo* steroid synthesis is exceptionally preserved. However, before onset of oestrus the steroidogenic capacity of perCL is temporarily limited. Production and reception of PGs is independent of the CL stage and ovarian cycle and is associated with maintaining CL function. High levels of PGE_2 in perCL might be important to fulfil the luteotrophic requirements for functional and structural CL persistence in lynxes. Felids exhibit no $PGF_{2\alpha}$-associated luteolytic mechanism in the absence of pregnancy, *i.e.*, luteal production of $PGF_{2\alpha}$ is not involved in cyclic luteal regression in cats. Likewise, regulation of the temporal functional CL regression by luteal $PGF_{2\alpha}$ in lynxes is negligible. In cats, $PGF_{2\alpha}$ synthesised by the placenta exhibits a likely role in prepartal luteal regression.

Perspective

It remained unclear which factors cause the shift in onset of luteal regression in the pregnant and non-pregnant luteal phase in cats and what initiates temporary regression at prooestrus in lynxes. Through differential expression studies (transcriptomics, proteomics) unknown luteotrophic and luteolytic factors might be identified. The question why cat CL undergo functional and structural luteal regression, whereas perCL in lynxes reduce their function while preserving the structural integrity, stays unanswered. Investigations on the bobcat, which phylogenetically is the most common ancestor of cats and lynxes, could enhance the validity of the present comparative studies, because despite perCL, it exhibits a polyoestrous cycle. The identification of potential luteotrophic and luteolytic factors in the presented studies allowed making first assumptions on their contribution to the feline CL lifespan. By functional cell culture studies, these assumptions could be evaluated by elucidating their influence on feline luteal cells *in vitro*. Prospectively, the basic research on the unique reproduction of lynxes can support the establishment of protocols for artificial ovulation induction. This can be implemented in adaptive conservation management plans to restore wild populations of Iberian lynxes.

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Selbstständigkeitserklärung

Hiermit erkläre ich, die Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze keinen entsprechenden Doktorgrad. Ich erkläre, dass ich die Dissertation oder Teile davon nicht bereits bei einer anderen wissenschaftlichen Einrichtung eingereicht habe und dass sie dort weder angenommen, noch abgelehnt wurde. Ich erkläre die Kenntnisnahme der dem Verfahren zugrunde liegenden Promotionsordnung der Lebenswissenschaftlichen Fakultät der Humboldt-Universität zu Berlin vom 5. März 2015. Weiterhin erkläre ich, dass keine Zusammenarbeit mit gewerblichen Promotionsberatern stattgefunden hat und dass die Grundsätze der Humboldt-Universität zu Berlin zur Sicherung guter wissenschaftlicher Praxis eingehalten wurden.

Berlin, 08.09.2015 Lina Zschockelt

Appendix

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Corpora lutea of pregnant and pseudopregnant domestic cats reveal similar steroidogenic capacities during the luteal life span

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Corpora lutea of pregnant and pseudopregnant domestic cats reveal similar steroidogenic capacities during the luteal life span

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ABSTRACT

In domestic cats, luteal phases of pregnancy and pseudopregnancy (non-pregnant luteal phase) differ in the course and level of plasma progesterone (P4). Therefore, we assumed differences in luteal steroidogenic capacities. Here we present a comprehensive analysis of intraluteal steroid biogenesis in the domestic cat. We quantitatively measured relative mRNA levels of steroidogenic acute regulatory protein (STAR), cytochrome P450 oxidases (CYP), hydroxysteroid dehydrogenases (HSD), steroid reductase (SRD) and enzymes involved in sulfoconjugation of steroids, i.e. sulfotransferase (SULT) and sulfatase (STS). Protein expression was analysed by Western Blot for HSD3B. Additionally, intraluteal steroid contents were determined. During the pseudopregnant luteal phase, expression of STAR ($p = 0.005$), HSD3B1 ($p < 0.0001$), CYP19A1 ($p < 0.0001$) and HSD17B7 ($p = 0.008$) decreased from formation of the *corpus luteum* (CL) onwards. HSD3B protein expression was highest in the development/maintenance stage of CL and declined during the subsequent luteal phase of pregnancy and pseudopregnancy. This was in accordance with decreasing intraluteal levels of P4, oestrogens and androgens. In contrast, expression of SRD5A1 ($p < 0.001$) increased with progression through stages of the pseudopregnant CL, being indicative of P4 metabolism via an alternate pathway to dihydrotestosterone (DHT). Compared to the formation stage, expression of SULT1E1 was higher in all other luteal stages of pseudopregnancy ($p = 0.004$), implying a potential sulfoconjugation of oestrogens. Expression of CYP11A1 and CYP17A1 was unaffected by the luteal stage ($p > 0.05$), suggesting a permanent capacity of cat CL to convert progestogens via androgen and oestrogen pathways. In general, mRNA expression profiles of steroidogenic enzymes during the pregnant luteal phase reflected the pseudopregnancy profiles. Intraluteal oestrogen ($p < 0.0001$) and androgen ($p = 0.008$) levels were higher in the formation stage compared to the following luteal stages of pseudopregnancy. Concentrations of P4 were higher in the development/maintenance compared to the regression stages ($p = 0.01$). We conclude that cat CL of the same histomorphological stage are characterised by identical steroidogenic capacities independently of an on-going pregnancy.

1. Introduction

Domestic cats (*Felis silvestris* forma *catus* Linnaeus, 1758) exhibit a coitus-induced luteal phase [1,2], although spontaneous ovulation without cervical or vaginal stimulation was confirmed as well [3,4]. The luteal phase in domestic cats is characterised by plasma progesterone (P4) elevations, reflecting the formation of functional *corpora lutea* (CL) on the ovary. Depending on the mating success, two types of luteal phases exist: one associated with pregnancy and a sterile luteal phase, often referred to as pseudopregnancy. The plasma P4 and oestradiol (E2) profiles during pregnancy and the pseudopregnant luteal phase have been

Abbreviations: CA, *corpus albicans*; CL, *corpus luteum*; CYP, cytochrome P450 oxidase; d/m, development/maintenance; DHT, dihydrotestosterone; E2, oestradiol; er, early regression; f, formation; HSD, hydroxysteroid dehydrogenase; lr, late regression; P4, progesterone; PP, pseudopregnancy; PR, pregnancy; SRD, steroid reductase; STAR, steroidogenic acute regulatory protein; STS, sulfatase; SULT, sulfotransferase.

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extensively investigated by blood sampling [1,5–9]. Within the first five days after mating, plasma E2 abruptly declines from preovulatory peak values to baseline and only slightly rises prior to parturition [5,6]. The levels of plasma E2 during pseudopregnancy are in a similar range compared to pregnancy [5]. The origin of preimplantative and prepartal E2 elevations remained unexplained, but follicular and luteal cells have been discussed as possible sources [1]. Plasma P4 profiles of pregnant and pseudopregnant cats proceed similarly until day 10–12 *post coitum* (*p.c.*) coinciding with time point of implantation [10]. Thereafter, plasma P4 levels are considerably higher in pregnant females, reaching peak values at day 21, followed by a constant decrease until parturition at day 65 ± 4 [5]. In the pseudopregnant luteal phase, plasma P4 already becomes basal after 40–45 days [7]. Assuming that plasma P4 levels reflect functional luteal phases in cats, the pseudopregnant phase lasts about two thirds of the respective gestational period. Shortened luteal phases have already been documented in pseudopregnant rabbits [11] and rats [12]. Other carnivores, like mink [13] and ferret [14], reveal luteal phases of pregnant and pseudopregnant females with similar lengths, whereas in the dog, CL can retain their function beyond normal time of parturition (80 versus 65 days) [15].

It has long been assumed that placental P4 contributes to the variation in plasma P4 levels in pregnant versus pseudopregnant cats. The placental capacity to convert pregnenolone to P4 near term [16], as well as intraplacental P4 content and expression of steroidogenic enzymes, have been subsequently proven [17,18]. However, differences between plasma P4 profiles in pregnant and pseudopregnant cats remained unexplained, as placental steroid profiles do not reflect plasma levels of P4 and E2 [17]. Furthermore, ovariectomies lead to a rapid decrease of plasma P4 and in some cases to abortion [8,9,19], providing evidence for luteal origin of plasma P4. Mechanisms for pregnancy maintenance after ovariectomy are still unknown, but might be supported by local P4 production by the feline placenta.

According to histomorphology and steroid hormone levels, the luteal tissue of cats undergoes severe transformations with progression through the stages of formation, development/maintenance, regression and the *corpus albicans* [20]. Little is known, however, about the underlying mechanisms of steroidogenic transformations during the luteal life span in cats. Generally, the conversion of cholesterol to active steroids within steroidogenic cells is catalysed by steroidogenic enzymes. Steroidogenic enzymes belong mainly to cytochrome P450 oxidases (CYP) and hydroxysteroid dehydrogenases (HSD); the steroid reductases (SRD) are unrelated to these families (Fig. 1; for review see Miller [21] and Ghayee and Auchus [22]).

The *de novo* steroid biogenesis requires transport of cholesterol through the mitochondrial membrane by the steroidogenic acute regulatory protein (STAR) [23]. Cholesterol is then converted to pregnenolone by the cholesterol side-chain-cleavage enzyme (CYP11A1). The oxidation of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids (e.g. pregnenolone to P4, androstenediol to testosterone) is catalysed by 3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerases (HSD3B). A steroid 17-alpha-monooxygenase (CYP17A1) converts pregnenolone and P4 to intermediates for testosterone and oestrogen synthesis. C19-androgens are aromatised to C18-oestrogens by P450 aromatase (CYP19A1). Different 17β-hydroxysteroid dehydrogenases (HSD17B) convert androstenedione to testosterone, as well as oestrone to E2, and vice versa [24]. Steroid-5-alpha-reductase isozymes (SRD5A) are involved in steroid metabolism by converting 3-oxo (3-keto), $\Delta^{4,5}$ C19/C21 steroids to 5α-stereoisomers, e.g. testosterone to dihydrotestosterone (DHT) [25] and progesterone to 5α-reduced progestogens [26]. Steroid sulphates are derived from sulfonation of steroids by sulfotransferase enzymes, whereby oestrogens are sulfonated by the cytosolic SULT1E1 [27]. The conformational changes of oestrogens by sulfoconjugation can be reversed by hydrolysis of steroid sulphates to native steroids by a sulfatase (STS) [21].

The aim of our study was to provide the first comprehensive analysis of intraluteal steroid biogenesis pathways in the domestic cat. Based on different plasma P4 profiles during pregnancy and pseudopregnancy [5], we assumed differences in luteal steroidogenic capacities. We quantitatively measured relative mRNA levels of steroidogenic acute regulatory protein, cytochrome P450 oxidases, hydroxysteroid dehydrogenases, steroid reductase and enzymes involved in sulfoconjugation of steroids. These data were supplemented by investigating protein expression of HSD3B. The steroid content in different luteal stages was determined as well. This study was conducted to contribute to fundamental knowledge on luteal function and to provide insight into the mechanisms regulating luteolysis in felids.

2. Materials and methods

2.1. Tissue collection

Ovaries and uteri of domestic cats were collected from local animal shelters and clinics after ovariohysterectomy/ovariectomy, whereby time point of ovulation was unknown for each animal. Ovaries were dissected for CL isolation. Day of pregnancy was determined according to (i) the stage of embryos flushed from the oviduct (<day 5 *p.c.*; [28]), (ii) the diameter of the gestation chamber (<day 20 *p.c.*; [29]) or (iii) to foetal crown-rump length (>day 20 *p.c.*; [30]). In absence of embryos or foetuses, a pseudopregnancy was assumed. Stages of pseudopregnancy were established based on the histomorphology of CL (routine haematoxylin/eosin staining) and by comparison to existing literature, as well as to the observed course of pregnancy [20]. Accordingly, CL of pregnant and pseudopregnant cats were allocated to five sequential luteal stages: formation (f), development/maintenance (d/m), early regression (er) and late regression (lr). For pregnancy this included day 2–5 (f), day 14–36 (d/m), day 38–39 (er) and day 48–63 (lr), respectively PP1 (f), PP2 (d/m), PP3 (er) and PP4 (lr) for pseudopregnancy. *Corpora albicantia* (CA) were not assigned to either pregnant or pseudopregnant luteal phases, since they could have been remnants of functional CL after parturition and weaning [31] or of a pseudopregnancy. Due to limitation of luteal tissue, histological, hormonal and molecular analyses were not performed on the same CL. However, comparability was ensured by random selection of CL with same appearance from one ovary for either fixation in Bouin's solution (histological analysis) or in liquid nitrogen (hormone, mRNA and protein studies).

2.2. Sequence analysis

Total RNA was isolated from feline reproductive tissues according to the innuSPEED Tissue RNA/innuPREP DNase I Digest Kit (*HSD17B7*; Analytik Jena AG, Jena, Germany) or the Precellys Tissue RNA/peqGOLD DNase I Digest Kit (*STAR*, *HSD17B2*, *SRD5A1*, *SULT1E1*, *STS*; PEQLAB Biotechnologie GmbH, Erlangen, Germany). Reverse transcription of total RNA into single-stranded cDNA (ss cDNA) was performed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Schwerte, Germany). For the polymerase chain reaction (PCR) primers were purchased from BioTeZ Berlin Buch GmbH (Berlin, Germany). Primers were based on predicted (*HSD17B7*) or published (*STAR*) *Felis catus* gene sequences. Sequences of *HSD17B2*, *SRD5A1*, *SULT1E1* and *STS* were not annotated in GenBank at the beginning of the study. Therefore, primers for their amplification were derived from consensus sequences after a multiple species sequence alignment (CLC

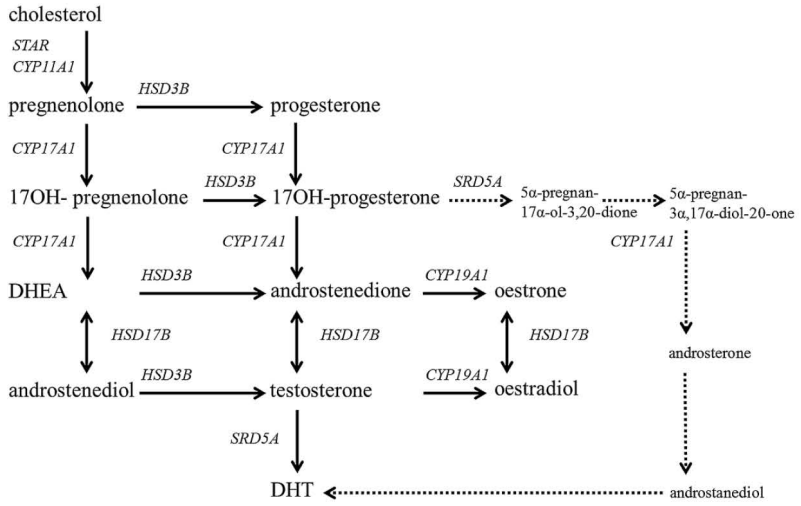


Fig. 1. Conventional (—) and alternate (- -) pathways of steroid biogenesis. STAR (steroidogenic acute regulatory protein), CYP11A1 (cholesterol side-chain-cleavage enzyme), HSD3B (3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerase), CYP17A1 (steroid 17-alpha-monooxygenase), CYP19A1 (P450 aromatase), HSD17B (17β-hydroxysteroid dehydrogenase), SRD5A (steroid-5-alpha-reductase). Modified according to Miller [21] and Ghayee and Auchus [22].

Sequence Viewer 6.7, CLC bio, Aarhus, Denmark) or from sequence comparisons of different species with the cat whole genome shotgun sequence (GenBank ID: AANG02000000), using the Basic Local Alignment Search Tool (National Center for Biotechnology Information, Maryland, USA). Primer information is listed in Table 1. Based on feline ss cDNA templates of luteal, ovarian or placental origin, partial or complete cDNA sequences were amplified using the Expand High FidelityPLUS PCR system (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), as described by Braun et al. [32]. The PCR conditions were: 94 °C for 2 min; followed by 35 cycles of denaturation (94 °C) for 45 s (*HSD17B7*, *SRD5A1*) or 60 s (*STAR*, *HSD17B2*, *SULT1E1*, *STS*), annealing for 45 s (*HSD17B7*, *SRD5A1*) or 60 s (*STAR*, *HSD17B2*, *SULT1E1*, *STS*), elongation (72 °C) for 60 s (*HSD17B7*, *SRD5A1*), 75 s (*STAR*, *HSD17B2*, *SULT1E1*) or 120 s (*STS*); and a final elongation at 72 °C for 7 min. For *HSD17B2*, *SRD5A1*, *SULT1E1* and *STS* the purified PCR products were ligated to the pJET 1.2 vector (Thermo Scientific) and transformed in JM109 cells (Promega GmbH, Mannheim, Germany). The *HSD17B7* product was ligated into the pCR4-TOPO TA vector and transformed in TOP10 cells (both Life Technologies GmbH, Darmstadt, Germany). The PCR product of *STAR* was based on a Eurasian lynx (*Lynx lynx*) template. Following ligation (pCR4-TOPO TA vector) the product was transformed into DH5α cells (both Life Technologies GmbH). Positive clones were sequenced by the Services in Molecular Biology GmbH (Dr M. Meixner, Brandenburg, Germany). Predicted and identified coding DNA sequences of PCR products or clones were aligned using ClustalW2 (European Bioinformatics Institute, Cambridge, Great Britain). Sequence information for other feline genes investigated in this study was described earlier by Braun et al. [17] for *CYP11A1* (JN165033), *CYP17A1* (JN172941), *HSD3B1* (JN127378), aromatase-wild type (*CYP19A1-WT*, GU306147), aromatase-deletion (*CYP19A1-Δ*, GU306148) and *HSD17B1* (JN165032).

2.3. mRNA expression studies

Up to 22 mg of luteal tissue was placed in homogenisation tubes (100 µl RNA lysis buffer, 1.4/2.8 mm ceramic beads). Tissues were disrupted in a Precellys 24 tissue homogenizer for 2 × 25 s at 5000 rpm (PEQLAB Biotechnologie GmbH). The total RNA was extracted using the innuSPEED Tissue RNA/innuPREP DNase I Digest Kit (Analytik Jena AG) or the Precellys Tissue RNA/peqGOLD DNase I Digest Kit (PEQLAB Biotechnologie GmbH). Concentration and purity of RNA were assessed with the NanoDrop ND-1000 (PEQLAB Biotechnologie GmbH). Additionally RNA quality and integrity were validated by microfluidic

Table 1
Sequences of PCR primers used for sequence analysis and expression studies, annealing temperatures, and product sizes.

GenBank ID[a]	Species	Primer sequence 5′–3′	T_A (°C)	Product size (bp)	Use
STAR KF831344[a]	*Lynx lynx*	STAR Fw: CTT GGT TCT CRG CTG GAA G STAR Rv: CAG CCA TCC CTT GAG GTC	53	579	a
		STAR qFw: AGC TTG TGG AGC ACA TGG AA STAR qRv: TGA TTC TGC AGC CAA CTC GT	61.5	116	b
HSD17B2 KC344427[a]	*Felis catus*	HSD17B2 Fw: GAG CAC ACA AAG GTG CAG G HSD17B2 Rv: GTT CCA AGA CTA ACA CTC CT	53	1234	a
		HSD17B2 qFw: CAT GGC AGG AGG GGT CC HSD17B2 qRv: TTC CAC ATT TCA CTC GTG CC	61.5	172	b
HSD17B7 KF831336[a]	*Felis catus*	HSD17B7 Fw: CAA AGG TTT CAG AGA TTA GAC T HSD17B7 Rv: GTC TTC ATC TAG GTC CAT CTT	55	644	a
		HSD17B7 qFw: ATG TTC TCC ACA GCT GAA GG HSD17B7 qRv: GCA TTG CGA GAC GAT GTC	58.5	175	b
SRD5A1 KC344428[a]	*Felis catus*	SRD5A1 Fw: AGG AGC TGC CCT CGC TG SRD5A1 Rv: TCT CAA GGT ACC ACT GGT G	53	553	a
		SRD5A1 qFw: GTC ACT GAT CCC CGA TTT CTA SRD5A1 qRv: CCA CCT CCC CAA AGT AGT TG	59	174	b
SULT1E1 JX983095[a]	*Felis catus*	SULT1E1 Fw: GCA GCC TAA AAC CTG AGT G SULT1E1 Rv: GTG AGG AAC GAT GAG AAG AA	53	1164	a
		SULT1E1 qFw: CGG AAC GCC AAA GAT GTT G SULT1E1 qRv: CCA GGA ACC ATA AGG CAC TTG	61.5	131	b
STS JX983094[a]	*Felis catus*	STS Fw: GCT GAC GAC CTT GGC ATC STS Rv: CTC GGT CAC ACT GGC AAG	53	1609	a
		STS qFw: GGA AAT GGA CTG GAG CGT T STS qRv: ACC GAA GAA TGC CAG GAA C	60.5	212	b

bp, base pair; fw, forward; rv, reverse; T_A, annealing temperature; a, used for sequence analysis; b, used for expression studies.
[a] All gene sequence information analysed in this study.

analysis using the Bioanalyzer (Agilent Technologies Deutschland GmbH, Boeblingen, Germany); RIN values for the analysed samples were above 7.0. One to five μg of each isolated RNA sample were reverse transcribed into ss cDNA in a 40 μl reaction volume using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). A no-reverse transcription control was included to check for genomic DNA contamination. For quantitative real-time PCR (qPCR) intron-spanning primers were based on sequences identified in this study (Table 1). Primer sequences, product sizes and qPCR conditions for *CYP11A1*, *CYP17A1*, *HSD3B1*, *CYP19A1-WT+Δ*, *CYP19A1-Δ* and *HSD17B1* were presented by Braun et al. in a study where two *CYP19A1* isoforms – wild type (WT) and deletion (Δ) – were detected in the cat placenta [17]. Therefore, amplification of *CYP19A1-WT+Δ* and *CYP19A1-Δ* in the CL was done using the same forward-primer, but different reverse-primers. The mRNA level of *CYP19A1-WT* (later referred to as *CYP19A1*) was calculated by subtracting the *CYP19A1-Δ*- from the *CYP19A1-WT+Δ*-quantities. The qPCR was conducted using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Munich, Germany) as published by Braun et al. [17]. Shortly, diluted ss cDNA (4 μl, corresponding to 2 or 10 ng of total RNA for genes of interest, respectively 4 ng for reference genes) were analysed in a 10 μl reaction volume including SsoFast EvaGreen Supermix (Bio-Rad Laboratories GmbH). The qPCR conditions were: 98 °C for 2 min (*CYP19A1-WT+Δ*; Δ: 95 °C) and 40 cycles of 98 °C for 8 s (*CYP19A1-WT+Δ*; Δ: 95 °C) and 8 s at different annealing temperatures (Table 1). Quantification of qPCR products was carried out with the CFX Manager Software 1.6 (Bio-Rad Laboratories GmbH). Serial recombinant plasmid DNA dilutions were used for calibration. For normalisation, fragments of reference genes (REF) were amplified as described previously for *GLS* (glutaminase; JQ424891), *TBP* (TATA box binding protein; JQ424890), *RPS7* (ribosomal protein S7; NM_001009832) [17] and *BACT* (β-actin; AB051104), *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase; NM_001009307) and *B2MG* (beta-2 microglobulin; NM_001009876) [33]. Finally *GLS*, *TBP* and *BACT* were validated as optimal REF in feline CL with the qbasePLUS software (Biogazelle, Zwijnaarde, Belgium; [34]). A multiple normalisation factor was calculated for individual CL referring to Vandesompele et al. [35].

2.4. Western Blot analysis

Protein homogenates of luteal tissue were prepared with a Speed-Mill. The lysis buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.5% Triton X-100, 2 mM EDTA) was supplemented with a protease inhibitor cocktail according to the manufacturer's instructions (Roche Diagnostics Deutschland GmbH). Tissue was homogenised with 150 μl lysis buffer/5 mg tissue in innuSPEED Lysis Tubes P (2.4/2.8 mm ceramic beads) using a Speed-Mill PLUS (both Analytik Jena AG) for 3 × 30 s under precooled conditions. Homogenates were centrifuged (14,000 × g, 4 °C, 30 min). Protein concentration in the supernatant was determined with the NanoDrop ND-1000 (PEQLAB Biotechnologie GmbH). 20 μg of protein lysates in sample buffer (50 mM Tris–HCl pH 6.8, 1% SDS, 0.125 M DTT, 12.5% glycerol, 0.01% bromophenol blue) were heated for 5 min at 95 °C. Samples were separated on 12% SDS-polyacrylamide gels and transferred to 0.2 μm nitrocellulose membranes (Whatman Protran BA83, GE Healthcare GmbH, Solingen, Germany) for Western Blot analysis. After transfer the membrane was incubated for 1 h with blocking solution (1% BSA and 1% milk powder in PBS, 0.05% Tween-20) and incubated overnight with mouse α-HSD3B (37-2) (1:400, sc-100466; Santa Cruz Biotechnology, Inc., Heidelberg, Germany). The membrane was washed with T-PBS (PBS with 0.2% Tween-20) and incubated for 1 h with secondary antibody, with subsequent washing in T-PBS and PBS. Secondary antibody was goat α-mouse POD (1:10.000, Sigma–Aldrich Chemie GmbH, Munich, Germany). The VersaDoc Imaging System 4000 MP and the Quantity One 1-D Analysis Software Version 4.6.9 (both Bio-Rad Laboratories GmbH) were used to detect and analyse chemiluminescence signals developed with ECL Prime (GE Healthcare GmbH). The detection signal of each band was depicted as intensity × mm.

2.5. Hormone analysis

For steroid hormone extraction from luteal tissue 8–68 mg tissue and 100 μl PBS were combined and transferred into homogenisation tubes (1.4/2.8 mm ceramic beads) and disrupted (2 × 25 s at 5500 rpm) in a Precellys 24 tissue homogenizer (PEQLAB Biotechnologie GmbH). Aliquots of 20 μl of homogenate (with added 0.5 ml PBS) were two times extracted with 2 ml tert-butyl methyl ether (TBME)/petroleum ether (PE; v/v, 30/70). By shaking for 30 min, freezing at −80 °C for 15 min and decantation of the organic phase, the remaining water phase was collected for a second extraction step. Subsequently, the two decanted extracts were combined and evaporated with N_2 at 54 °C. The remainder was finally dissolved in 0.5 ml of 40% methanol. For determination of intraluteal androgen concentrations, 20 μl of each extract were analysed with an in-house enzyme immunoassay (EIA). The antibody was kindly provided by Prof H.H.D. Meyer (TU München, Freising-Weihenstephan, Germany) and was raised in rabbits immunised against 17α-OH-testosterone-HSRSA. The cross reactivity of the antibody to different androgens was described previously: 4-androsten-17β-ol-3-one (testosterone) −100%, 5α-androstan-17β-ol-3-one (dihydrotestosterone) −13.6%, 5α-Androst-2-en-17β-ol −1.5%, and <0.1% for 5α-androstan-3β-ol-17-one (epiandrosterone), 5α-androstan-3α-ol-17-one (androsterone), 5α-androst-2-en-17-one and 4-androsten-17β-ol-3-one sulphate (testosterone sulphate) [36]. Testosterone-3-CMO-peroxidase was used as enzyme conjugate. Serial dilutions of a testosterone standard and of a feline luteal sample revealed parallelism. Inter- and intra-assay coefficients of variation for two biological samples with low and high concentrations were 10 and 16% ($n = 6$) and 8 and 2% ($n = 10$), respectively. For P4 and oestrogen analysis 20 μl and 10 μl of extracts were used as previously described by Carnaby et al. [37]. A commercial P4 antibody (Sigma P1922, raised in rats) and 4-pregnen-3,20-dione-3-CMO-peroxidase as enzyme conjugate were used [38]. Oestrogen analysis was performed based on a polyclonal antibody (rabbit) against 1,3,5(10)-estratrien-3,17β-diol-17-HS-BSA and 1,3,5(10)-estatrien-3,17β-diol-17-HS peroxidase conjugate [39].

2.6. Statistical analysis

Due to animal health issues, castration of pregnant cats at the animal shelters and clinics occurred seldom, only in the context of population control of feral cats (capture–release action). Thus, CL stages of pregnancy were obtained randomly and prepartal stages were rarely available to the authors of the present study. Consequently, results for pregnant cats were only analysed descriptively. Exclusively for pseudopregnant CL stages, the non-parametric Kruskal–Wallis rank sum test was applied to determine the influence of different luteal stages on the relative mRNA and steroid levels. The Wilcoxon rank sum test was used for *post hoc* pairwise comparisons (p-value adjustment: Benjamini–Hochberg). *Corpora albicantia* were excluded from this analysis. Statistical analysis was done with The R software package (R: a language and environment for statistical computing, version 2.15.2, Vienna, Austria). Values of $p < 0.05$ were considered significant.

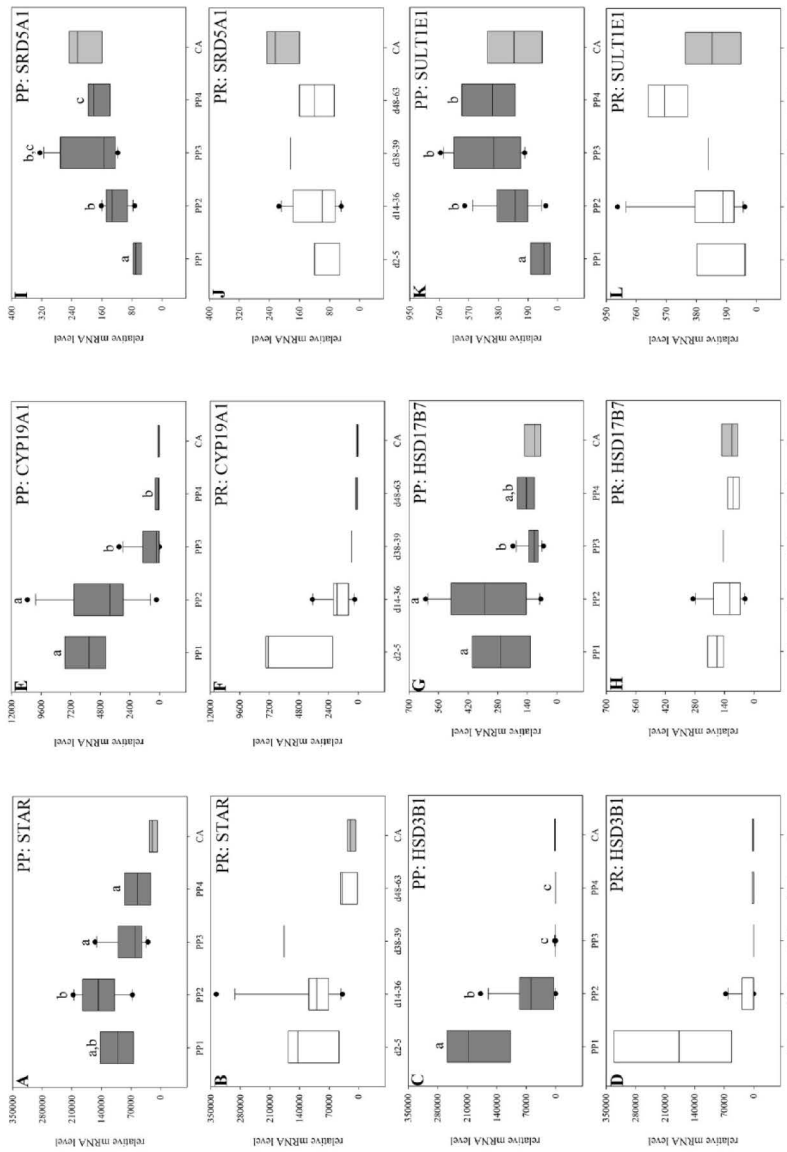


Fig. 2. Relative mRNA levels of steroidogenic enzymes in different luteal stages of pregnant and pseudopregnant domestic cats. (A–B) *STAR*, (C–D) *HSD3B1*, (E–F) *CYP19A1*, (G–H) *HSD17B7*, (I–J) *SRD5A1* and (K–L) *SULT1E1* expression. PP, pseudopregnancy; PR, pregnancy; f, formation; d/m, development/maintenance; er, early regression; lr, late regression; CA, *corpus albicans*. The Kruskal–Wallis rank sum test was applied for the pseudopregnant luteal phase (CA-stage excluded).

3. Results

3.1. Feline gene sequences of steroidogenic enzymes

In this study, *Felis catus* (partial) gene sequences were identified based on cDNA of the following steroidogenic enzymes: *HSD17B2* (KC344427), *HSD17B7* (KF831336), *SRD5A1* (KC344428), *SULT1E1* (JX983095) and *STS* (JX983094). For *HSD17B2* we identified a complete coding DNA sequence (cds) of 1234 bp, with two differences to the predicted *F. catus* sequence (XM_003998308). The identified gene sequence of *HSD17B7* consists of 644 bp, showing two nucleotide exchanges towards the prediction (XM_003999544.1), which do not alter the amino acid sequence. The partial cds of *SRD5A1* (553 bp) revealed four differences to the gene prediction (XM_003981575), but showed 100% similarity to the predicted protein. Also a complete cds of 1164 bp for *SULT1E1* was identified, with an additional 126 bp insert before the start codon and one substitution within the cds as compared to the predicted sequence (XM_003985305). Furthermore, a part of the coding region of *STS* (1609 bp) was identified with two differences from the predicted sequence (XM_004000254), but without an effect on the protein sequence. A partial *STAR* sequence of 579 bp was obtained based on a Eurasian lynx template (KF831344) revealing three substitutions in relation to the *F. catus* sequence (NM_001246196).

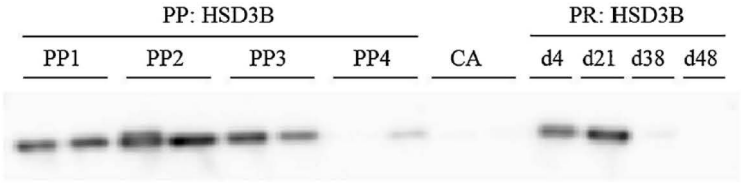
3.2. mRNA expression of steroidogenic enzymes at different luteal stages

Based on the qPCR results, intraluteal gene expression of the investigated steroidogenic enzymes was confirmed in each analysed luteal stage within both luteal phases (Fig. 2, Table 2). Only mRNA levels for *HSD17B2* were hardly detectable and therefore excluded from further analysis. During the pseudopregnant luteal phase statistically different mRNA levels were observed for six steroidogenic enzymes. Highest mRNA levels at formation stage (PP1) and/or development/maintenance (PP2) were revealed for *STAR* ($p = 0.005$; Fig. 2A), *HSD3B1* ($p < 0.0001$; Fig. 2C), *CYP19A1* ($p < 0.0001$; Fig. 2E), and for *HSD17B7* ($p = 0.008$; Fig. 2G), accompanied by a general decrease of the expression profile. By contrast, the relative mRNA levels of *SRD5A1* ($p < 0.001$; Fig. 2I) were highest during early (PP3) and late (PP4) regression, reflected in an overall increasing profile; in tendency this was also the case for *HSD17B1* ($p = 0.092$; Table 2). The last two enzymes further revealed relatively high expression in the CA-stage. Compared to formation (PP1), the expression of *SULT1E1* ($p = 0.004$; Fig. 2K) was higher in the developmental and regression stages (PP2–PP4). For *CYP11A1*, *CYP17A1* and *STS* the expression was unaffected by the luteal stage ($p > 0.05$; Table 2). Interestingly, mRNA levels were relatively high for *STAR*, *CYP11A1* and *CYP17A1* compared to other enzymes investigated. In tendency, the expression profiles of steroidogenic enzymes during the pregnant luteal phase (Fig. 2B, D, F, H, J and L) were reflected in the profile courses of the pseudopregnant luteal phase with relative mRNA levels being within same ranges for individual factors (Table 2). A detailed analysis of the *HSD3B1* pregnancy profile per day *p.c.* revealed that within the d/m-stage from day 14–21 the mRNA levels were comparable to those in the formation stage, whereby levels from day 23–36 reflected expression within the early regression stage. A similar variability of the *HSD3B1* expression within the d/m-stage was found for pseudopregnancy CL. This phenomenon of functional and structural decoupling was neither supported by histomorphology nor by other steroidogenic enzymes investigated in our study.

Table 2
Quantitative real-time PCR analysis of steroidogenic enzymes in different luteal stages of pregnant and pseudopregnant domestic cats.

	Formation	Development/maintenance		Early regression		Late regression		*C. albicans*
	PP1 ($n = 5$)	d2–5 ($n = 3$)	PP2 ($n = 12$)	d14–36 ($n = 11$)	PP3 ($n = 12$)	d38–39 ($n = 2$)	PP4 ($n = 8$)	d48–63 ($n = 3$)	CA ($n = 4$)
CYP11A1	480994 ± 273128	504197 ± 231295	565808 ± 134838	502990 ± 224813	676796 ± 319325	792790 ± 272624	676064 ± 413512	388439 ± 271174	362674 ± 214779
CYP17A1	20990 ± 17829	28940 ± 27617	4161 ± 3536	6992 ± 8386	13528 ± 25072	11867 ± 1567	10176 ± 9108	29079 ± 36216	56609 ± 77244
HSD17B1	74 ± 31	157 ± 127	88 ± 69	90 ± 38	107 ± 62	97 ± 79	146 ± 73	353 ± 239	275 ± 112
HSD17B2	7 ± 6	14 ± 15	1 ± 1	1 ± 1	1 ± 1	0	0	1 ± 1	0
STS	154 ± 97	157 ± 81	151 ± 81	117 ± 64	108 ± 56	135 ± 9	82 ± 38	122 ± 13	64 ± 39

Mean values ± standard deviations per luteal stage are depicted as relative mRNA levels referring to 1 ng original total RNA. CA, *corpus albicans*; PP, pseudopregnancy.



PP1	PP2	PP3	PP4	CA	d4	d21	d38	d48
53025; 61569	87872; 86782	66400; 41167	n. d.; 10768	1080; 1478	56009	71833	3706	n. d.

Fig. 3. Protein expression of HSD3B in different luteal stages of pregnant and pseudopregnant domestic cats. HSD3B (42 kDa) expression and quantity. PP, pseudopregnancy; PR, pregnancy; CA, *corpus albicans*; n.d., not detectable. Luteal tissue was homogenised and 20 μg of protein lysates were used for Western Blot analysis. Quantity of each band is depicted as intensity × mm.

Table 3
Enzyme immunoassay analysis of steroid hormones in different luteal stages of pregnant and pseudopregnant domestic cats.

	Formation		Development/maintenance		Early regression		Late regression		*C. albicans*
	PP1 ($n=6$)	d2, 4, 5 ($n=3$)	PP2 ($n=11$)	d19–36 ($n=6$)	PP3 ($n=10$)	d38, 39 ($n=2$)	PP4 ($n=7$)	d48 ($n=1$)	CA ($n=2$)
Progesterone	51 ± 48	18; 42; 39	88 ± 29	82 ± 25	36 ± 44	22; 20	31 ± 29	22	13; 3
Oestrogen	457 ± 724	419; 371; 142	83 ± 65	44 ± 48	11 ± 12	18; 11	9 ± 11	6	3; 3
Androgen	797 ± 851	145; 461; 193	143 ± 116	63 ± 24	68 ± 73	731; 42	108 ± 179	43	32; 13

Single values or mean values ± standard deviations per luteal stage are depicted as ng/g (oestrogen, androgen) or μg/g (progesterone) luteal tissue. CA, *corpus albicans*; PP, pseudopregnancy.

3.3. HSD3B protein expression at different luteal stages

As revealed by Western Blot analysis, intraluteal HSD3B protein expression was influenced by the different luteal stages (Fig. 3). Moderate signals of HSD3B expression (at about 42 kDa) were detected at the formation stage (PP1, day 4). Highest HSD3B expression was shown at the development/maintenance stage (PP2) and at day 21 of pregnancy. Intensity of signals for the early regression stage (PP3) was comparable to CL formation. Weak HSD3B expression was detected at day 38 of pregnancy, late regression (PP4) and the CA-stage, whereas no signals were identified at day 48.

3.4. Intraluteal steroid content at different luteal stages

Steroids were detectable in all CL via EIA (Table 3). The P4 concentrations were higher during development/maintenance (PP2, 88 ± 29 μg/g), compared to the regression stages (PP3, PP4; $p=0.01$). High P4 values were also detected at the developmental CL stage of pregnancy (82 ± 25 μg/g). Intraluteal oestrogen levels peaked during formation (PP1: 457 ± 724 ng/g) and were still high in PP2, compared to the regression stages PP3 and PP4 ($p<0.0001$). The changes in intraluteal oestrogen levels throughout pseudopregnancy were reflected in the histomorphological stages of pregnancy. Intraluteal androgens were also highest during formation (PP1: 797 ± 851 ng/g; e.g. day 4: 461 ng/g) and decreased with progression of the pseudopregnant luteal phase ($p=0.008$). All investigated steroids reached basal levels at the CA-stage.

4. Discussion

This study presents the first comprehensive analysis of intraluteal steroid biogenesis in the domestic cat. Based on histomorphological categorisation, we investigated expression of enzymes involved in steroid biogenesis and sulfoconjugation, as well as intraluteal steroid contents in different luteal stages of pregnant and pseudopregnant cats. During the luteal life span, synthesis of biologically active P4, oestrogens and androgens in the CL decreases, whereas the potential metabolism of these steroid hormones increases.

In previous studies, luteal activity in cats was mainly assessed by quantification of circulating steroids in ovarian/uterine venous blood [9] or in systemic/peripheral plasma [1,5–8] and by examination of the influence of ovariectomy on pregnancy maintenance [8,9,19]. Siemieniuch et al. were the first to investigate expression of STAR and HSD3B within the cat CL of pregnancy and pseudopregnancy [18]. But a comprehensive analysis of intraluteal steroid synthesis and metabolism was still missing. Taking previous publications into account, our study provided further evidence for the capability of cat CL to take up and convert cholesterol to active steroids. Thereby, *de novo* steroid biogenesis seems to be regulated via provision of cholesterol, since intraluteal mRNA expression was affected by the luteal stage for *STAR* (Fig. 2A), but not for *CYP11A1*. These findings reflect the situation in the dog, where intraluteal *STAR* expression constantly decreases from preimplantation until preparturition [40]. In contrast, Siemieniuch et al. described the luteal *STAR* expression in cats as strongly time-dependent for pregnancy with peak values at mid pregnancy (3–4 weeks *p.c.*), but not for pseudopregnant luteal phases [18]. Their group defined the beginning of luteal phases by silencing of oestrus behaviour and correlated their data to the week/day of on-going pregnancy/pseudopregnancy. We defined day of pregnancy according to the embryo/foetus development, but for pseudopregnant luteal phases no daily determination was possible. This partly hinders a direct comparison of the respective data sets. Future research on cats with known plasma hormone profiles will allow correlating the daily assessment provided by Siemieniuch et al. with our histomorphological approach. Though provision of cholesterol via STAR might decrease during luteal phases, our study demonstrated a sustained capacity of cat CL to convert progestogens via androgen and oestrogen pathways. Even in the CA-stage, *STAR* mRNA expression

was substantially high. It was recently proven that the STAR protein is expressed in feline CL during each luteal phase of pseudo-/pregnancy [18]. Moreover, our study revealed high expression of factors for initial cholesterol cleavage (*CYP11A1*) and intermediate steroid synthesis (*CYP17A1*) throughout the luteal phase (Table 2). Whereby regressing cat CL might still synthesise intermediates, CYP17A1 activity is lost in some species right after ovulation and luteinisation [41].

The decline in intraluteal P4, oestrogen and androgen levels (Table 3) during pseudo-/pregnancy was accompanied by a severe decrease of *HSD3B1* (synthesis of P4, intermediates for androgens and oestrogens; Fig. 2C–D), *CYP19A1* (oestrogen pathway; Fig. 2E–F) and *HSD17B7* (oestrogen synthesis; Fig. 2G–H) mRNA expression. The most profound decrease occurred at the transition from the development/maintenance towards the regression stage. Siemieniuch et al. revealed time-dependent *HSD3B* mRNA expression with elevations during the mid-luteal stages (3–4 weeks of pregnancy; days 10–15 of pseudopregnancy) and detected HSD3B proteins in luteal cells during each luteal phase via immunohistochemistry [18]. Our study showed highest *HSD3B1* mRNA expression already during the preimplantative CL formation of both luteal phases. This, together with the observed decrease of intraluteal *HSD3B1* expression from preimplantation until the preparturition period, resembles the situation in the dog [40]. Interestingly, mRNA expression profiles of *HSD3B1* in cat CL were only slightly consistent with intraluteal profiles of P4. However, initially low intraluteal and plasma P4 levels during CL formation and peak values around day 21 *p.c.* were mirrored by the course of HSD3B protein expression, as was equally revealed for pregnancy and pseudopregnancy by our study (Fig. 3). The following decline of intraluteal P4 and oestrogens from development/maintenance towards early regression was also in accordance with the respective decrease in HSD3B1 mRNA and protein expression.

It is worth noting, that the basal intraluteal P4 levels in the regression stages of pregnancy are in contrast to the prolonged elevation of plasma P4 until shortly before parturition [5]. It was shown that P4 can be used as a substrate for SRD5A1 in reproductive tissue via an alternate pathway to DHT; the yielded 5α-reduced progesterone metabolites are not biologically active regarding the binding to the P4 receptor (Fig. 1; [26,42]). Minjarez et al. therefore assumed that in mice, parturition at term is highly dependent on local tissue metabolism of P4 in the uterus [26]. Due to increasing intraluteal *SRD5A1* expression during pseudopregnancy, we propose a similar mechanism of P4 withdrawal in cat CL as well, suggesting intraluteal P4 levels are not solely regulated by HSD3B1.

Peak values of oestrogen occurred at CL formation in pregnancy and pseudopregnancy, followed by a strong decline towards CL development/maintenance with constant low levels till the end of the luteal life span (Table 3). The *CYP19A1* mRNA expression partly mirrored intraluteal oestrogen levels. Due to the limited availability and specificity of commercial antibodies, no cat-specific signal for CYP19A1 protein could be detected in our study. Intraluteal oestrogen contents can also be affected by conversion of oestrone to E2 via HSD17B1 [24]. During the luteal phase of rodents, E2 is mainly secreted by the CL, whereby HSD17B7, rather than HSD17B1, catalyses the final step of E2 synthesis [43]. Our study revealed opposing courses of *HSD17B1* (Table 2) and *HSD17B7* (Fig. 2G–H) expression profiles. We suggest that intraluteal HSD17B isoforms play a role in maintaining the steady-state synthesis of E2. Time-dependent expression of *SULT1E1* (Fig. 2K) further indicates sulfoconjugation of oestrogens within the cat CL. Sulfoconjugation inhibits the biological effect of steroids through conformational changes that hinder steroid sulphates from binding to their nuclear receptors [44]. It further facilitates transport of intermediates as supply for steroid biogenesis [45]. A directed activation of oestrogen-conjugates by desulfation seems negligible, due to steady-state expression of *STS* in the luteal phases (Table 2). We assume that intraluteal oestrogens locally support preimplantative CL formation in cats, suggesting a luteal-ovarian contribution to postovulatory plasma E2 elevations. The origin of prepartal E2 elevations in plasma [5] remains unresolved, since intraluteal oestrogen levels could only be measured until day 48 of pregnancy.

Except for the early regression stage of pregnancy, intraluteal androgen profiles mirrored oestrogen and *HSD3B1* profiles of both luteal phases. We assume that HSD3B1 and/or HSD17B limit the provision of testosterone as a direct precursor for E2 synthesis. Contrarily, withdrawal from E2 synthesis might occur by intraluteal testosterone metabolism to the more potent DHT via the SRD5A2 isozyme (Fig. 1; not investigated by our study). DHT and testosterone are mainly involved in formation of the prostate gland and male external genitalia [25]. However, aside being a precursor for oestrogen synthesis, in primates androgens were shown to modulate follicular development [46]. Moreover, testosterone revealed a direct luteotrophic effect on luteal function and lifespan in mice [47]. At the moment, regulation and biological role of androgen synthesis in feline luteal tissue remains speculative.

To conclude, feline CL are capable of *de novo* steroid biogenesis, demonstrated by the expression of all necessary steroidogenic enzymes. Our study could not reveal differences in steroidogenic capacities of CL during pregnant and pseudopregnant luteal phases based on the currently available data set. Almost identical intraluteal expression profiles of steroidogenic enzymes, as well as intraluteal contents of steroids, suggest that on-going loss of steroidogenic capacity similarly causes a functional demise of the CL. Nonetheless, as temporal differences during CL lifespans are depicted by different plasma P4 levels, future studies should address additional luteal factors that might cause variation in the time-dependent onset of luteolytic mechanisms in felids.

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Comparative analysis of intraluteal steroidogenic enzymes emphasises the functionality of fresh and persistent *corpora lutea* during pro- and metoestrus in the lynx

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Comparative analysis of intraluteal steroidogenic enzymes emphasises the functionality of fresh and persistent *corpora lutea* during pro—and metoestrus in the *lynx*

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ABSTRACT

European *lynx* species demonstrate an atypical ovarian cycle compared to other felids. The physiological persistence of *corpora lutea* (CLs), reflected in constantly elevated progesterone (P4) concentrations in serum, is thought to ensure a seasonal monooestrus. Moreover, the coexistence of CLs from a recent ovulation (freshCLs) and persistent CLs from previous years (perCLs) on the same ovary has been proven. We assume that perCLs in *lynxes* occur due to fundamentally different mechanisms of luteal regression. Our study presents a detailed analysis of steroidogenic enzymes and steroids in fresh and perCLs obtained from *Iberian lynxes* during metoestrus, and in perCLs obtained from *Eurasian lynxes* during prooestrus. By quantitative PCR we measured relative mRNA amounts of steroidogenic acute regulatory protein (*STAR*), cytochrome P450 oxidases (CYPs), hydroxysteroid dehydrogenases (*HSDs*) and a steroid reductase (SRD). Protein expression in CLs was investigated for CYP11A1, CYP17A1, CYP19A1 and HSD3B. Additionally, the intraluteal and serum steroid content was determined. During metoestrus, mRNA amounts of *STAR*, *CYP11A1*, *CYP19A1*, *HSD17B7* and *SRD5A1* were significantly higher in perCLs compared to freshCLs. Protein of CYP11A1 was detected independently of the CL age in metoestrus, but expression was less evident in prooestrous perCLs. The protein signal of CYP17A1 was strong in freshCLs and perCLs of metoestrus, but weak at prooestrus. The presence of CYP19A1 protein was confirmed in each stage of the CL. These findings contribute to the hypothesis that CLs from previous years might support freshly developed CLs for pregnancy maintenance. However, initiation of ovulation might require a functional down-regulation of perCLs prior to breeding. It is noteworthy that the *HSD3B1* mRNA amount was significantly elevated in fresh compared to perCLs (metoestrus). Accordingly, *HSD3B* protein was substantially present in freshCLs, whereas signals were literally absent in all perCLs. Elevated expression of HSD3B coincided with high intraluteal oestrogen concentrations in freshCLs; however, the enzyme pattern was less concordant with intraluteal P4 and androgen concentrations. Serum P4 concentrations of *Iberian lynxes* were constant between prooestrus and prolonged dioestrus. Moreover, constantly high serum oestrogen concentrations were measured during pro-, met- and prolonged dioestrus. The physiology of exceptionally high serum oestrogen concentrations outside the breeding season of *lynxes* merits further investigation. In conclusion our study supports the concept that the unique reproductive strategy of *lynxes* is directly linked to sustained intraluteal steroid biogenesis in persistent CLs.

Abbreviations: CL, *corpus luteum*; CYP, cytochrome P450 oxidase; EL, *Eurasian lynx*; freshCL, freshly formed CL; HSD, hydroxysteroid dehydrogenase; IL, *Iberian lynx*; P4, progesterone; perCL, persistent CL; SRD, steroid reductase; STAR, steroidogenic acute regulatory protein.

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1. Introduction

The *lynx* genus consists of four species of short-tailed cats (family *Felidae*) found in the forests of Europe, Asia and North America. The *Eurasian lynx* (*Lynx lynx* Linnaeus, 1758), the *Canada lynx* (*L. canadensis* Kerr, 1792) and the bobcat (*L. rufus* Schreber, 1777) are considered as *least concern* by the International Union for Conservation of Nature and Natural Resources (IUCN), whereas the *Iberian lynx* (*L. pardinus* Temminck, 1827) is declared as *critically endangered* [1]. Therefore, various attempts to protect this species from extinction have been initiated. An integrated species conservation plan links the *in situ* (*Iberian Lynx* Conservation Breeding Programme, ILCBP; Portugal, Spain) with the *ex situ* (Lince Andalucía—Population recovery of *Iberian Lynx* in Andalusia, EU LIFE Project) conservation efforts. The main goals of the ILCBP are to maintain a genetically well-managed captive population, supporting wild populations by *lynx* re-introduction [2–4]. Furthermore, the ILCBP provides the basis for research on reproductive physiology and associated technologies [5].

Captive population management of *Iberian* and *Eurasian lynxes* is already supported by the tools of pregnancy diagnosis and parturition prediction [6,7]. Moreover, non-invasive monitoring of hormone metabolites [8,9], as well as studies on steroid metabolism [10,11], have documented endocrine profiles of reproduction in female *lynxes*. As a central reproductive peculiarity, Iberian and *Eurasian lynxes* exhibit a non cat-like ovarian cycle, as shown by ultrasonographical and endocrinological analyses [12]. The Canada, the Iberian and the *Eurasian lynx* all reveal a strict seasonal monooestrous cycle [2,8,13,14] in contrast to the polyoestrous reproductive patterns of most other felids [15] including the bobcat [16,17] and the domestic cat (*Felis silvestris forma catus* Linnaeus, 1758; [18]).

As there exist no confounding species differences regarding reproduction, except for a moderate shift in the beginning of the mating season (Jan–Apr for *Eurasian lynx* [14], Jan–Feb for *Iberian lynx* [2]), the *Eurasian lynx* is commonly used as a model species to gain knowledge of the reproductive physiology of the critically endangered *Iberian lynx* [10,12,19]. Recently, *intra-vitam* longitudinal ultrasound studies of *Eurasian lynx* ovaries, supported by information on serum steroid concentrations, revealed the physiological persistence of *corpora lutea* (CLs) [19]. These persistent CLs are preserved structurally for at least two subsequent years and are assumed to be responsible for the almost constantly elevated progesterone (P4) concentrations in serum throughout the year, thus preventing ovulation and, presumably, ensuring the monooestrous cycle [12,19]. The observation that central and northern European populations of both captive and free-ranging *Eurasian lynxes* all show the same reproductive strategy suggests that there is no plasticity regarding this phenomenon [19]. The only difference was found concerning the beginning of the breeding seasons (oestrus occurred at the end of February until mid-March for captive *lynxes* and at the end of March for free-ranging *lynxes*), which is most likely latitude, and therefore delayed photoperiod, dependant [19]. Since luteal activity is observed outside the breeding season, in contrast to the typical anoestrus period of other felids, this activity was referred to as prolonged dioestrus (for a detailed description of the reproductive stages of the *Eurasian lynx* cycle see [19]). Histological characterisation and determination of intraluteal steroid content in *Eurasian lynx* further established the coexistence of freshly formed CLs of a recent ovulation (freshCLs) and persistent CLs of previous years (perCLs) on the same ovary [20–22]. Despite annual luteal activity, female *lynx* can regularly enter oestrus, and gestation is terminated by parturition, which is supposedly ensured by temporary functional regression of CLs prior to the onset of oestrus or near term [19].

In most mammals, CLs functionally and structurally regress at the end of the luteal phase in a process called luteolysis [23]. In pregnant and non-pregnant domestic cats, *e.g.* the structural transition from CL formation to the *corpus albicans* [24,25] correlates with decreasing plasma P4 concentrations [26–28]. Since this is reflected by an ongoing loss of intraluteal steroidogenic capacity [29,30], intraluteal steroids are potential factors for assessing luteal function in felids.

Regulation of *de novo* steroid biogenesis requires mobilisation and transfer of cholesterol by the steroidogenic acute regulatory protein (STAR) [31]. Basic pathways of conventional and alternate steroid biogenesis and steroid metabolism comprise conversion of cholesterol to pregnenolone, metabolism of pregnenolone into various intermediates and active steroids, and metabolism of steroid precursors and steroids [32] (Fig. 1). Most steroidogenic enzymes belong to cytochrome P450 oxidases, such as the

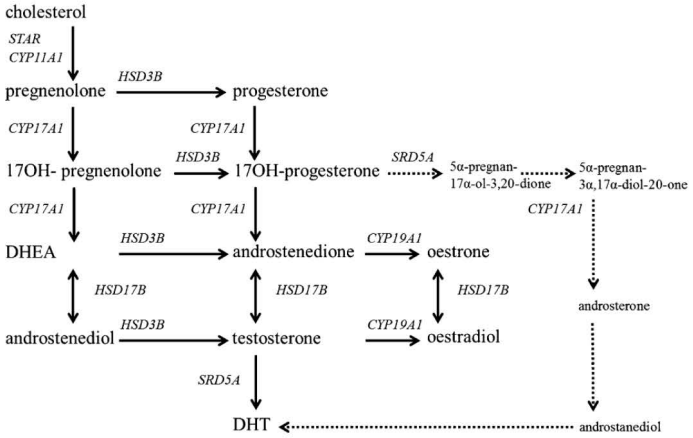

Fig. 1. Conventional (—) and alternate (- -) pathways of steroid biogenesis. STAR (steroidogenic acute regulatory protein), CYP11A1 (cholesterol side-chain-cleavage enzyme), HSD3B (3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerase), CYP17A1 (steroid 17-α-monooxygenase), CYP19A1 (P450 aromatase), HSD17B (17β-hydroxysteroid dehydrogenase), SRD5A (steroid 5-α-reductase), DHEA (dehydroepiandrosterone), DHT (dihydrotestosterone). Reproduced with permission from J Steroid Biochem Mol Biol, 2014. 144(Part B): p. 373–81 [29].

cholesterol side-chain-cleavage enzyme (CYP11A1), the steroid 17-α-monooxygenase (CYP17A1) and the P450 aromatase (CYP19A1), and the hydroxysteroid dehydrogenases, such as the 3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerases (HSD3B) and the 17β-hydroxysteroid dehydrogenases (HSD17B1, HSD17B2 and HSD17B7) [33]. Isozymes of steroid 5-α-reductase (SRD5A) convert steroids to more potent derivatives, such as testosterone to dihydrotestosterone [34], and are involved in steroid metabolism, *e.g.* by converting progesterone to 5α-reduced progestogens [35,36].

Our study aimed to investigate the steroidogenic capacity of freshly formed and persistent *Iberian lynx* CLs at metoestrus (day 7 *post-coitum* (*p.c.*); freshCLs and perCLs), when perCLs might also be under the influence of freshCLs. Additionally, perCLs obtained from *Eurasian lynxes* at the beginning of the breeding season prior to mating (prooestrus) were included in the analysis. The present investigations were conducted as a contribution towards elucidating the unique reproductive strategies of female *lynxes*.

2. Materials and Methods

2.1. Animals and tissue collection

Studies on CLs of Iberian and *Eurasian lynxes* were strictly limited by their conservation status and the collection of tissue occurred only on occasion. Within the ILCBP, ovariohysterectomy of two captive *Iberian lynxes* was performed seven days *p.c.* (February 2013), for medical and management reasons. As determined by oviduct flushing, morulae were found in one animal (*Iberian lynx* 1, IL1), whereas unfertilised oocytes were found in the second female (*Iberian lynx* 2, IL2). As our previous study demonstrated that CLs of pregnant and non-pregnant domestic cats reveal similar steroidogenic capacities during the luteal life span [29], the stage of CLs of both *lynxes* was equally determined as pre-implantation. The CLs were isolated immediately after the surgery. The ovaries of both animals revealed the coexistence of freshCLs (recent ovulation, CL formation stage; IL1 $n_{CLqPCR/EIA} = 3/2$, IL2 $n_{CLqPCR/EIA} = 5/3$, for description of CLs stages see [24]) and perCLs (previous oestrus cycles, CL maintenance stage; IL1 $n_{CLqPCR/EIA} = 8/4$, IL2 $n_{CLqPCR/EIA} = 6/4$).

Fresh *post-mortem* ovarian tissue in five free-ranging *Eurasian lynxes* (*Eurasian lynx* 1–5, EL1–EL5) was collected at the beginning of the breeding season prior to mating (prooestrus; February 2011) from fresh carcasses obtained from controlled, legal hunting during the national hunting quota for management purposes in Norway. Isolated CLs were characterised as persistent (EL1 $n_{CLqPCR/EIA} = 4/2$, EL2 $n_{CLqPCR/EIA} = 4/2$, EL3 $n_{CLqPCR/EIA} = 2/1$, EL4 $n_{CLqPCR/EIA} = 4/2$, EL5 $n_{CLqPCR/EIA} = 4/1$) and revealed histological signs of early regression (data not shown).

All stages of *lynx* CLs were determined by histomorphology using established domestic cat CL parameters [24]. Each CL was dissected and pieces were fixed in Bouin's solution (immunohistochemistry) or placed in RNAlater RNA Stabilization Reagent (RNA isolation; Qiagen GmbH, Hilden, Germany) or Allprotect Tissue Reagent (*Eurasian lynx*, hormone and protein analyses; Qiagen GmbH) or liquid nitrogen (*Iberian lynx*, hormone and protein analyses). The various solutions were applied to adequately preserve the tissue obtained under the different conditions for transport to the laboratory.

Additional captive *Iberian lynx* females from the ILCBP were included for blood sampling to analyse serum steroid concentrations: prooestrus (Dec–Jan), metoestrus (Feb), pregnancy (Mar) and prolonged dioestrus (Apr–Nov). The respective n-numbers are depicted in Table 3. Blood samples were taken by venipuncture during annual veterinary check-ups and in the case of pregnant females non-invasively (peripheral blood) by blood-sucking bugs (*Dipetalogaster maxima*), as described and validated earlier [6,37].

2.2. Sequence analysis

Total RNA isolation from reproductive tissue and reverse transcription to cDNA was performed as previously described [29]. Most sequences of the analysed genes were not annotated in GenBank prior to this study. Primers for the polymerase chain reaction (PCR) were therefore based on gene sequences of the domestic cat or on consensus sequences derived from multiple species sequence alignments (CLC Sequence Viewer 6.7, CLC bio, Aarhus, Denmark). Primers were purchased from BioTez Berlin Buch GmbH (Berlin, Germany; Table 1). Partial or complete cDNA sequences were amplified from luteal, ovarian or placental tissue using the Expand High FidelityPLUS PCR system (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), as defined earlier [38]. The PCR was conducted at 94 °C for 2 min followed by 35 cycles of denaturation (94 °C) for 20 sec (*B2 M*), 45 sec (*BACT, HSD17B7*), 30 sec (*CYP11A1, CYP17A1, GLS, RPS7, TBP*) or 60 sec (*GAPDH, HSD17B2, SRD5A1*), annealing for 20 sec (*B2 M*), 30 sec (*CYP11A1, CYP17A1, GLS, RPS7*), 45 sec (*BACT, HSD17B7, TBP*) or 60 sec (*GAPDH, HSD17B2, SRD5A1*), elongation (72 °C) for 30 sec (*B2 M*), 45 sec (*BACT, CYP11A1, CYP17A1, GLS, RPS7*), 60 sec (*HSD17B7*), 70 sec (*TBP*), 75 sec (*GAPDH, HSD17B2*) or 100 sec (*SRD5A1*) and a final elongation at 72 °C for 7 min. For amplification of *HSD17B1* cDNA, the TrueStart Taq DNA Polymerase kit (Life Technologies GmbH, Darmstadt, Germany) was used with 95 °C/2 min; 35 cycles of 95 °C/30 sec, 55 °C/30 sec, 72 °C/45 sec; and 72 °C/7 min. Purified PCR products of *B2 M, BACT, HSD17B2, RPS7* and *TBP* were ligated to the pJet 1.2 vector (Thermo Scientific, Schwerte, Germany), followed by transformation into JM109 cells (Promega GmbH, Mannheim, Germany). Products of *HSD17B7* and *SRD5A1* were ligated into the pCR4-TOPO TA vector and transformed into TOP10 cells (both from Life Technologies). For some genes (*CYP11A1, CYP17A1, GAPDH, GLS, HSD17B1*), only *lynx*-specific PCR products were sequenced, but not cloned. Sequencing of clones or purified PCR products was performed by the Services in Molecular Biology GmbH (Dr M. Meixner, Ruedersdorf, Germany). *Lynx*-specific sequence information on *HSD3B1* (*L. pardinus*; JN127377), *CYP19A1*-wild-type (*L. pardinus*; GU306149), *CYP19A1*-deletion (*L. pardinus*; GU306150) and *STAR* (*L. lynx*; KF831344) was published previously [29,39].

2.3. Quantitative PCR

Homogenisation of luteal tissue (14–26 mg), total RNA extraction and reverse transcription to cDNA was done according to a previous study [29]. Quality and integrity of RNA were assessed using the Bioanalyzer (Agilent Technologies Deutschland GmbH, Boeblingen, Germany); RNA integrity number (RIN) values were above 6.1. Additionally, RNA concentration and purity were validated by the NanoDrop ND-1000 (Peqlab Biotechnologie GmbH, Erlangen, Germany). No-reverse transcription controls were included to test for genomic DNA contamination. Intron-spanning primers for quantitative PCR (qPCR) were designed according to sequences identified in the present study (Table 1) or used as in previous studies [29,39,40]. For *CYP19A1* expression analysis, separate qPCR were conducted due to the occurrence of two enzyme isoforms: CYP19A1-wild-type (WT) and CYP19A1-deletion (Δ) [41]. Products of *CYP19A1*-WT + Δ and *CYP19A1*-Δ were amplified using the same forward primer, but different reverse primers. The mRNA amount of *CYP19A1*-WT (=*CYP19A1*) was calculated by subtraction of *CYP19A1*-Δ quantities from *CYP19A1*-WT + Δ as previously validated [39]. For qPCR, diluted cDNA (4 or 10 ng of total RNA for genes of interest; 4 ng for

Table 1
Lynx-specific gene sequences of steroidogenic enzymes and reference genes.

GenBank ID	Species	Primer sequence 5′#5→9#	T_A (°C)	Product (bp)	use
HSD3B1 JN127377*	*L. pardinus*	*Hsd3b1* qFw: TTG GTG GAG GAG AAG GAC C *Hsd3b1* qRv: CGG TGT GGA TGA TGA CTG A	58	181	b
HSD17B1 KR014121	*L. lynx*	*Hsd17b1* Fw: CGA GCT CCT CTC CCC ATG *Hsd17b1* Rv: GAG GAA GAC CTC GGT CAC C	55	704	a
HSD17B2	*L. pardinus*	*Hsd17b2* Fw: GAG CAC ACA AAG GTG CAG G *Hsd17b2* Rv: GTT CCA AGA CTA ACA CTC CT	53	1189	a
KJ394957		*Hsd17b2* qFw: CAT GGC AGG AGG GGT CC *Hsd17b2* qRv: CCA CAT TTC ACT CGT GCC C	61.5	172	b
HSD17B7 KF831337	*L. lynx*	*Hsd17b7* Fw: CAA AGG TTT CAG AGA TTA GAC T *Hsd17b7* Rv: GTC TTC ATC TAG GTC CAT CTT	55	644	a
CYP11A1 KM982686	*L. lynx*	*Cyp11a1* Fw: CGT CTR TTC AGR ACC AAG AC *Cyp11a1* Rv: GAC CTT CCT CCA GCA CC	55–50	760	a
CYP17A1	*L. lynx*	*Cyp17a1* Fw: CTG TGG GCR CTG CAT CAC A *Cyp17a1* Rv: GGG ATG CTA GGT CAG CC	55–50	324	a
KM502566		*Cyp17a1* qFW: GAC CAG TTC ATG CCC GAA C *Cyp17a1* qRv: GGA CCT CCA GGT CGA ACC T	61.5	175	b
SRD5A1 KF831345	*L. lynx*	*Srd5a1* Fw: AGG AGC TGC CCT CGC TG *Srd5a1* Rv: TCT CAA GGT ACC ACT GGT G	51	493	a
B2M	*L. pardinus*	*B2m* Fw: CTT GGT CCT GCT CGG RCT *B2m* Rv: TTA SAK GTC TCG ATC CCA CTT	51	301	a
KM458619		*B2m* qFw: CTA TCT GTC CCA CCT GGA TG *B2m* qRv: GTC CAG TCC CTG TTG AAG G	58	191	b
BACT	*L. pardinus*	*Bact* Fw: CAT CCT GAC CCT CAA GTA C *Bact* Rv: TCA TGA TGG AGT TGA AGG	51	625	a
KM458620		*Bact* qFw: GAG CAG GAG ATG GCC ACG *Bact* qRv: CTC GTG GAT GCC ACA GGA	62	159	b
GAPDH KM458621	*L. pardinus*	*Gapdh* Fw: CTG GTC ACC AGG GCT GCT *Gapdh* Rv: CCA TGA GGT CCA CCA CCC	53	640	a
GLS JX993350	*L. pardinus*	*Gls* Fw: GAT GGC TGG CAA TGA ATA CG *Gls* Rv: CAG CAC ATC ATG CCC ATG AC	55	371	a
RPS7	*L. pardinus*	*Rps7* Fw: GCC ATG TTC AGT TCG AGC G *Rps7* Rv: GTC TAC AAC TGA AAC TCT GGG	55	550	a
JX993349		*Rps7* qFw: CCT GGA GGA CTT GGT TTT CC *Rps7* qRv: CCT TGC CCG TGA GCT TCT	61	164	b
TBP JX993351	*L. pardinus*	*Tbp* Fw: ATG GAT CAG AAC AAC AGC CTG *Tbp* Rv: GCA GGA GTA CGT TAA CAG CC	56–51	970	a

*, gene sequence information analysed in a previous study [39]; a, used for sequence analyses; b, used for expression studies; bp, base pair; fw, forward; rv, reverse; T_A, annealing temperature.

reference genes) was analysed with the CFX96 Real-Time PCR Detection System using the SsoFast EvaGreen Supermix (both from Bio-Rad Laboratories GmbH, Munich, Germany; for detailed description see [39]). Conditions for qPCR were: 98 °C for 2 min (*CYP19A1*-WT + Δ; Δ: 95 °C) and 40 cycles of 98 °C for 8 sec (*CYP19A1*-WT+Δ; Δ: 95 °C) and 8 sec at different annealing temperatures (Table 1). Information on qPCR primer sequences, product sizes and qPCR conditions for *CYP11A1*, *GAPDH*, *GLS*, *HSD17B1*, *HSD17B7*, *SRD5A1*, *STAR* and *TBP* was given previously [29,39,40]. The qPCR products were quantified with the CFX Manager Software 1.6 (Bio-Rad Laboratories GmbH) using recombinant plasmid DNA for calibration. Fragments of different reference genes were amplified for normalisation (Table 1): beta-2 microglobulin (*B2 M*), β-actin (*BACT*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), glutaminase (*GLS*), ribosomal protein S7 (*RPS7*) and TATA box binding protein (*TBP*). The qbasePLUS software (Biogazelle, Zwijnaarde, Belgium; [42]) identified *BACT*, *GAPDH*, *RPS7* and *TBP* as optimal reference genes for analysis of *lynx* CLs. A multiple normalisation factor was calculated for individual CLs referring to Vandesompele et al. [43].

2.4. Western Blot

Protein homogenates of luteal tissue were prepared as described before [29]. The protein concentration was measured with the NanoDrop ND-1000 (Peqlab Biotechnologie GmbH). For Western Blot analysis, lysates (20 μg of protein) were separated on 12 % SDS-polyacrylamide gels and thereafter transferred to 0.2 μm nitrocellulose membranes (Whatman Protran BA83, GE Healthcare GmbH, Solingen, Germany; see also [29]). The membranes were incubated overnight with mouse anti-3β-HSD (1:400, sc-100466; Santa Cruz Biotechnology, Inc., Heidelberg, Germany). The secondary antibody was goat anti-mouse IgG (1:10.000, A 3682; Sigma-Aldrich Chemie GmbH, Munich, Germany). Chemiluminescence signals were developed with ECL Prime (GE Healthcare GmbH) and detected using the VersaDoc Imaging System 4000 MP (Bio-Rad Laboratories GmbH). To compare signals for Iberian and *Eurasian lynxes* obtained from separate Western Blots, an *Iberian lynx* sample (freshCL) was run in parallel on the gel for *Eurasian lynxes*.

2.5. Immunohistochemistry

For immunohistochemical analysis, sections of fixed and embedded luteal tissue (Bouin's solution, paraffin, 3 μm) were mounted on microscope slides (Superfrost Plus, Thermo Scientific) and treated as published earlier [38]. Briefly, slides were deparaffinised in Roti Histol (Carl Roth GmbH, Karlsruhe, Germany) and rehydrated in ethanol. Antigenic sites were enhanced by incubation in boiling citrate buffer (11 mM, pH 6.0). Endogenous peroxidase activity was quenched by incubation in 3 % H_2O_2 with methanol. Nonspecific binding of the primary antibody was reduced by blocking with 5 % BSA in PBS. Sections were incubated overnight with primary antibody (Table 2). Control slides to test for specificity of staining were incubated with blocking solution instead of primary antibody. Slides were subsequently washed and incubated with secondary antibody (Table 2). The immunoperoxidase colour reaction was developed with diaminobenzidine substrate chromogen solution (Dako Deutschland GmbH, Hamburg, Germany). Sections were counterstained with haematoxylin, dehydrated in ethanol and covered with Roti Histokitt (Carl Roth GmbH) and cover-slips. Slides were analysed with an Axioplan microscope combined with a ProgRes C10 plus camera (both Carl Zeiss MicroImaging GmbH, Goettingen, Germany) and the Cell^P Soft Imaging Software (Olympus Soft Imaging Solutions GmbH, Muenster, Germany).

Table 2
Description of primary and secondary antibodies used for Western Blot and immunohistochemistry analyses.

Antibody	Antigen	Host	Type	Dilution	Source
anti-3β-hydroxysteroid dehydrogenase	Human	Mouse	Monoclonal	1: 100, IH 1: 400, WB	sc-100466; Santa Cruz Biotechnology, Inc.
anti-aromatase cytochrome P450	Mouse	Rabbit	Polyclonal	1: 100, IH	provided by Dr. Nobuhiro Harada
anti-cholesterol side-chain cleavage cytochrome P450	Bovine	Rabbit	Polyclonal	1: 5000, IH	[52]
anti-17α-hydroxylase cytochrome P450	Porcine	Rabbit	Polyclonal	1: 3000, IH	[53]
goat anti-mouse IgG-HRP	Mouse-IgG	Goat	Secondary antibody	undiluted, IH	K4000; Dako Deutschland GmbH
goat anti-rabbit IgG-HRP	Rabbit-IgG	Goat	Secondary antibody	undiluted, IH	K4002; Dako Deutschland GmbH
anti-mouse IgG (Fab specific)-peroxidase	Mouse-IgG	Goat	Secondary antibody	1: 10,000, WB	A 3682; Sigma-Aldrich Chemie GmbH

IH, immunohistochemistry; WB, Western Blot.

2.6. Enzyme-linked immunosorbent assay

Homogenisation of luteal tissue (9–65 mg) and steroid hormone extraction was done as previously described [29]. Briefly, aliquots of luteal homogenates were twice extracted with tert-butyl methyl ether (TBME)/petroleum ether (PE). Extracts were eventually dissolved in 40% methanol. For determination of intraluteal androgen concentrations, extracts were analysed with an in-house enzyme immunoassay (EIA) as published earlier [29]. The antibody was kindly provided by Prof. H.H.D. Meyer (TU München, Freising-Weihenstephan, Germany) and was raised in rabbits immunised against 17α-OH-testosterone-HSRSA. The cross-reactivity of this antibody to different androgens was described by Jewgenow et al. [44]. Intraluteal P4 and oestrogens concentrations were analysed as described by Carnaby et al. [20]. A commercial P4 antibody (Sigma P1922, raised in rats) was used [10]. Analysis of oestrogen concentrations was performed based on a rabbit polyclonal antibody against 1,3,5(10)-estratrien-3,17β-diol-17-HS-BSA [45]. For serum hormone analysis, ingested blood was withdrawn from the stomachs of the blood-sucking bugs. The serum was separated after centrifugation. Steroid extraction was done likewise with TBME/PE [12]. The P4 and oestrogen assays were previously validated for the *lynx* [8,10].

2.7. Statistical analysis

Due to the difficulty in obtaining larger amounts of samples in these endangered species, the number of replicates was limited and statistical analysis was only possible to some extent. For the two *Iberian lynxes*, the non-parametric Mann–Whitney *U*-test was applied to determine the influence of fresh and persistent stage of CLs on the relative mRNA amount per *lynx*. The R software package (R: A language and environment for statistical computing, version 2.15.2, Vienna, Austria) was applied for statistical analysis (values of $p < 0.05$ were considered significant). Other than this, the distribution of relative mRNA amounts (n_{CL}) and intraluteal steroid concentrations (n_{animal}/n_{CL}) is depicted in vertical Box Plots plotting data as median and 25th and 75th percentiles (SigmaPlot 10.0, Systat Software GmbH, Erkrath, Germany). Concentrations of serum steroids are presented as single values or mean values ± standard deviation (n_{animal}/n_{serum}).

3. Results

3.1. mRNA expression of steroidogenic enzymes

In this study, partial and complete gene sequences of *L. lynx* or *L. pardinus* were identified for different steroidogenic enzymes: *CYP11A1* (KM982686), *CYP17A1* (KM502566), *HSD17B1* (KR014121), *HSD17B2* (KJ394957), *HSD17B7* (KF831337), and *SRD5A1* (KF831345). Specific gene sequences for *L. pardinus* reference genes were submitted to GenBank as well: *B2 M* (KM458619), *BACT* (KM458620), *GAPDH* (KM458621), *GLS* (JX993350), *RPS7* (JX993349) and *TBP* (JX993351; Table 1).

Significant differences in intraluteal gene expression of almost all investigated steroidogenic enzymes between freshCLs and perCLs were revealed for *Iberian lynx* (IL1, IL2) by qPCR analysis (Fig. 2 A–H). Since relative mRNA amounts for *HSD17B2* were almost undetectable, this gene was excluded from the analysis. Compared to all other enzymes investigated, mRNA amounts of *CYP11A1* (Fig. 2B) were especially high in perCLs of *Iberian lynxes*. The mRNA amounts of *STAR* (IL1: $p = 0.0121$; IL2: $p = 0.0043$; Fig. 2A), *CYP11A1* (IL1: $p = 0.0121$; IL2: $p = 0.0043$; Fig. 2B), *CYP19A1* (IL1: $p = 0.0485$; IL2: $p = 0.0043$; Fig. 2D), *HSD17B7* (IL1: $p = 0.0121$; IL2: $p = 0.0043$; Fig. 2G) and *SRD5A1* (IL1: $p = 0.0121$; IL2: $p = 0.0043$; Fig. 2H) were higher in perCLs of each individual Iberian *lynx* compared to freshCLs of the same animal. For *CYP17A1* (Fig. 2C) and *HSD17B1* (Fig. 2F) no significant effect of the luteal stage on intraluteal gene expression was determined; however, there was high variability within each group of CLs and animal. By contrast, only in the case of *HSD3B1* (Fig. 2E), the mRNA amounts were substantially elevated in fresh compared to perCLs of *Iberian lynxes* (IL1: $p = 0.0121$; IL2: $p = 0.0043$).

In general, medians of relative mRNA amounts revealed high variation between perCLs of individual *Eurasian lynxes* (EL1–EL5) and were intermediate compared to freshCLs and perCLs of *Iberian lynxes* (Fig. 2A–H). Only for *HSD3B1*, relative mRNA amounts were similar between perCLs of Eurasian and *Iberian lynxes*, whereas

Table 3
Serum steroid content determined in female *Iberian lynxes* at different reproductive stages.

	Prooestrus (Dec–Jan)	Metoestrus (Feb)[a]	Pregnancy (Mar)[b]	Dioestrus (Apr–Nov)
progesterone	14.0 ± 6.6	9.4; 27.2	17.6 ± 10.7	8.4 ± 5.3
[ng/ml]	(11/11)	(2/2)	(9/19)	(40/87)
oestrogen	1397 ± 659	769; 994	–	1199 ± 695
[pg/ml]	(11/11)	(2/2)		(36/76)

Serum steroid content determined by enzyme-linked immunosorbent assay is depicted as single values or mean values ± standard deviation. (x/y), (n_{animal}/n_{serum});
[a] *Iberian lynx* IL1 and IL2;
[b] Serum samples obtained by blood-sucking bugs (*Dipetalogaster maxima*) as described earlier [6,37].

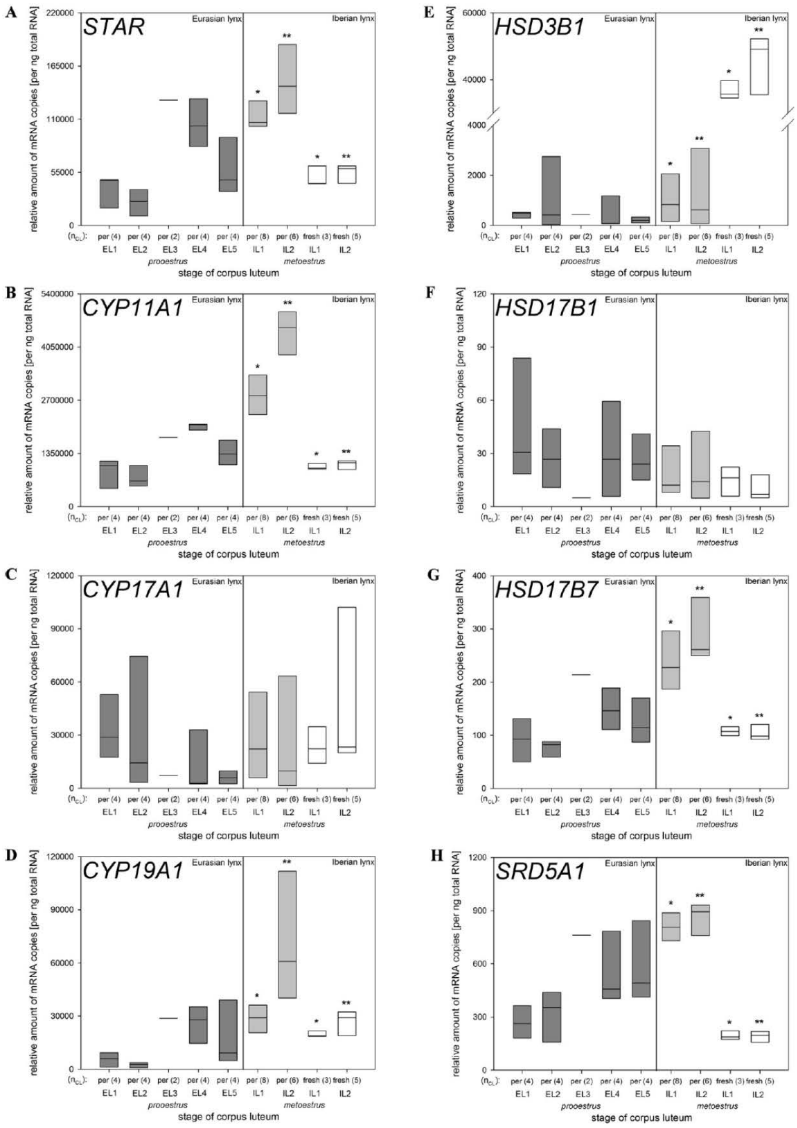

Fig. 2. Quantitative PCR (qPCR) analysis of mRNA expression of steroidogenic enzymes in corpora lutea (CLs) of *Eurasian lynxes* (prooestrus) and *Iberian lynxes* (metoestrus). Morphologically assessed stages of CLs were persistent (EL1–EL5), persistent (IL1, IL2) and fresh (IL1, IL2). Differences in intraluteal gene expression were revealed between fresh and perCLs of *Iberian lynx*. Relative amounts of mRNA copies are copy numbers determined by a calibration curve in qPCR that were afterward normalised with a factor deduced from qPCR results of reference genes. Values refer to 1 ng original total RNA. For *Iberian lynxes*, the non-parametric Mann–Whitney *U*-test was applied to determine the influence of CLs stages on relative mRNA amount. Data were plotted per individual *lynx* and CL stage as n_{CL}. *, $p < 0.05$ for IL1; **, $p < 0.05$ for IL2; EL, *Eurasian lynx*; IL, *Iberian lynx*; per, persistent.

CYP11A1 amounts were similar for perCLs of prooestrus and freshCLs of metoestrus.

3.2. Cellular localisation and protein expression of steroidogenic enzymes

As revealed by immunohistochemistry, staining for protein expression of CYP11A1, CYP17A1, CYP19A1 and HSD3B (Figs. 3 and 4) was localised to luteal cells. The staining was associated with cytoplasm, but not with cell nuclei.

In metoestrous *Iberian lynxes* signals of CYP11A1 expression were detectable independently of the luteal stage (freshCL or perCL; Fig. 3B and C). Also expression of CYP17A1 appeared similarly intense in freshCLs and perCLs (Fig. 3 E and F). The HSD3B expression signal was strongest in freshCLs (Fig. 4B and C). Additionally, Western Blot analysis of HSD3B protein expression indicated its dependence on the luteal stage. Signals for HSD3B expression (≈42 kDa) were confirmed solely within freshCLs of *Iberian lynxes* (Fig. 4D and E). Immunoreactivity of CYP19A1 was

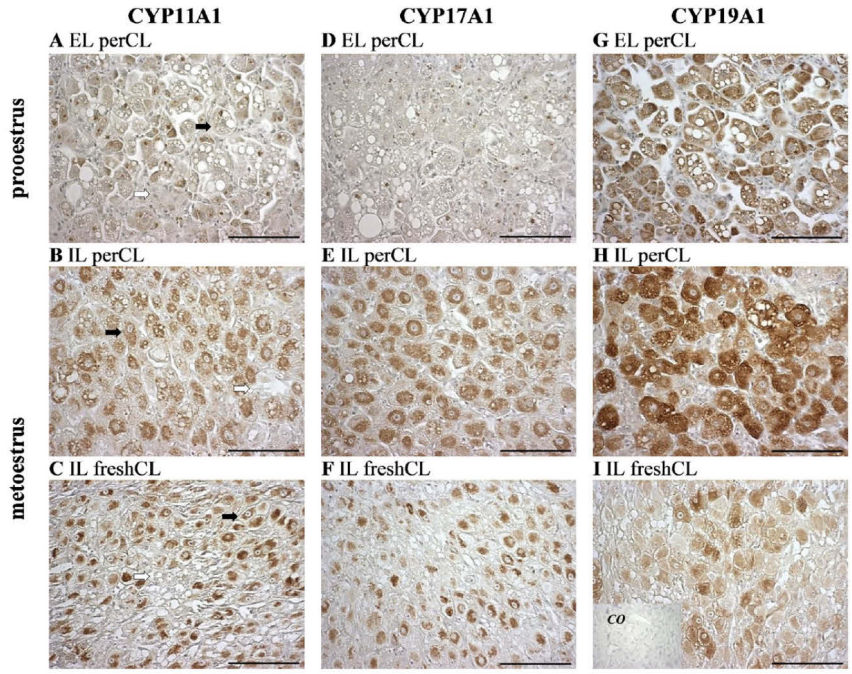

Fig. 3. Immunohistochemistry analysis of protein expression of steroidogenic enzymes in *corpora lutea* (CLs) of *Eurasian lynxes* (prooestrus) and *Iberian lynxes* (metoestrus). Morphologically assessed stages of CLs were persistent (EL), persistent (IL) and fresh (IL). Cell types identified were luteal (➔) and non-luteal cells (⇨). Pronounced CYP11A1 (B + C) and CYP17A1 (E + F) expression was detected at day 7 *p.c.* independently of the luteal stage, whereas protein expression was low in perCLs during prooestrus (A + D). CYP19A1 protein (G–I) was confirmed in all CL stages. Specificity of staining was controlled by incubation with blocking solution instead of primary antibody. Scale bar = 100 μm. CL, *corpus luteum*; CO, control (goat anti-rabbit IgG); EL, *Eurasian lynx*; IL, *Iberian lynx*; per, persistent.

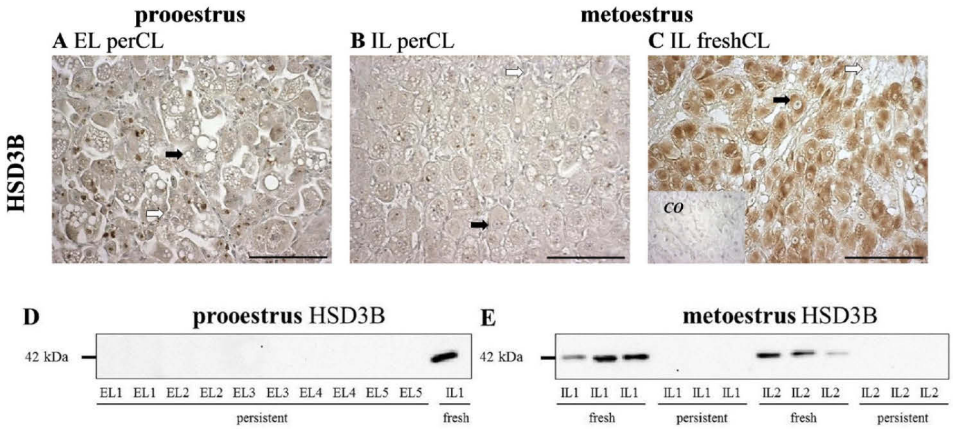

Fig. 4. Immunohistochemistry and Western Blot analyses of HSD3B protein expression in *corpora lutea* (CLs) of *Eurasian lynxes* (prooestrus) and *Iberian lynxes* (metoestrus). Morphologically assessed stages of CLs were persistent (EL), persistent (IL) and fresh (IL). Cell types identified were luteal (➔) and non-luteal cells (⇨). Strong HSD3B protein expression was localised in luteal cells of freshCLs (C), but was less abundant in the different perCLs (A + B). Specificity of staining was controlled by incubation with blocking solution instead of primary antibody. By Western Blot, HSD3B expression (≈42 kDa) was only confirmed in freshCLs of *Iberian lynxes* (D + E). Scale bar = 100 μm. CL, *corpus luteum*; CO, control (goat anti-mouse IgG); EL, *Eurasian lynx*; IL, *Iberian lynx*; per, persistent.

confirmed in each analysed CL of the two *lynx* species (Fig. 3G–I) but with strongest signals in metoestrous perCLs.

In prooestrous perCLs of *Eurasian lynxes* only weak signals of CYP11A1 (Fig. 3A), CYP17A1 (Fig. 3D) and HSD3B (Fig. 4A) expression were detected.

3.3. Intraluteal and serum steroid content

Using EIA, intraluteal and serum concentrations of steroids were detectable at each of the different luteal and reproductive stages. Statistical analysis was not possible due to low sample size.

During metoestrus of *Iberian lynxes*, median concentrations of intraluteal P4 were numerically higher in fresh compared to perCLs (Fig. 5A). Concentrations of serum P4 at day 7 *p.c.* were 9.4 ng/ml for IL1 and 27.2 ng/ml for IL2 (February) and serum P4 of *Iberian lynxes* in tendency revealed constant concentrations throughout the year (Table 3). The intraluteal oestrogen concentrations appeared to be substantially higher in fresh compared to perCLs of metoestrus (Fig. 5B). Serum oestrogen concentrations on day 7 *p.c.* were 769 pg/ml in IL1 and 994 pg/ml in IL2. Oestrogen concentrations tended to be constant throughout the annual cycle as well (Table 3). The median androgen concentrations were numerically higher in fresh than perCLs of metoestrous *Iberian lynxes* (Fig. 5C).

The lowest intraluteal median concentrations of P4 (Fig. 5A), oestrogen (Fig. 5B) and androgen (Fig. 5C) were measured in the prooestrous perCLs of *Eurasian lynxes*.

4. Discussion

Our study presents a detailed analysis of conventional steroidogenic enzymes and intraluteal steroid content determined in CLs of the two European *lynx* species. We propose an exceptional preservation of *de novo* steroid biogenesis in perCLs of *lynxes* during metoestrus and other cycle stages, as deduced from the steroidogenic activity of metoestrous perCLs of *Iberian lynxes*. However, before onset of a new breeding season the steroidogenic function of perCLs might be temporarily limited, as deduced from the low expression of key steroidogenic enzymes in prooestrous perCLs of *Eurasian lynxes*.

Our group initially analysed the intraluteal steroid content of CLs during the annual cycle in *Eurasian lynxes* [20]. However, detailed investigations of intraluteal steroid biogenesis with a focus on fresh and persistent luteal tissue were not previously conducted. The present study supports the assumption, that the *de novo* steroidogenic capacity and the capacity to provide intermediates for synthesis of active steroids is limited prior to onset of oestrus in perCLs of *Eurasian lynx* (prooestrus, February). In contrast to considerably high relative mRNA amounts of *STAR*, *CYP11A1* and *CYP17A1* the protein expression of CYP11A1 (initial cleavage of cholesterol to pregnenolone [32]) and CYP17A1 (qualitative regulation of steroid biogenesis [33]) was low in perCLs at the beginning of the breeding season. Noteworthy, expression of these two key steroidogenic enzymes was more evident within perCLs of metoestrous *Iberian lynxes*. Possible unknown cross-species variations causing this observation cannot be excluded, but were considered unlikely. Apart from that, synthesis of active steroids in prooestrous perCLs might be further restricted by the limited HSD3B expression. According to mRNA amounts, CYP19A1 protein expression was abundant in luteal cells of perCLs of prooestrus, but moderate amounts of *HSD3B1* mRNA were not reflected by the almost absent protein expression. The intraluteal concentrations of P4, oestrogen and androgen measured in the prooestrous perCLs were relatively low.

In a previous study we showed, that plasma PGFM ($PGF_{2\alpha}$ metabolite) concentrations slightly increase, concomitant with a P4 decline, at prooestrus in *Eurasian lynxes* [19]. The PGFM peak most likely induces the functional luteolysis of perCLs [19]. In accordance with the present study, these findings favour the general assumption that in *lynxes* successful initiation of folliculogenesis and ovulation requires functional down-regulation of perCLs prior to a new breeding season [5,19]. The temporary reduction of steroid synthesis in prooestrous perCLs was especially demonstrated by the intraluteal oestrogen concentrations. This might be a prerequisite to regularly enter oestrus despite constantly elevated P4 plasma concentrations [19].

Our findings suggest that in metoestrous *Iberian lynxes* fresh and perCLs reveal similar capacities for *de novo* steroid biogenesis and synthesis of steroid intermediates. Despite the elevated gene expression of *STAR* and *CYP11A1* in perCLs, pronounced protein signals of CYP11A1 were detected in both CL types. Also the protein expression of CYP17A1 was confirmed in both fresh and perCLs. In domestic cats STAR is constantly expressed throughout the luteal phase [29,30] and the intraluteal expression of CYP17A1 was proposed to be maintained far beyond ovulation and luteinisation [29], which is in contrast to many other species [46]. We assume that the expression of enzymes catalysing initial and intermediate steps of steroid biogenesis is preserved in perCLs of *lynxes* outside the pre-breeding season as well.

As during metoestrus there were no differences in the protein expression of enzymes associated with *de novo* steroid biogenesis, our results imply that synthesis of active steroids in fresh and perCLs of *Iberian lynxes* is mainly regulated by HSD3B. Diverging from expression patterns of all other steroidogenic enzymes studied, *HSD3B1* mRNA amounts within freshCLs were on average

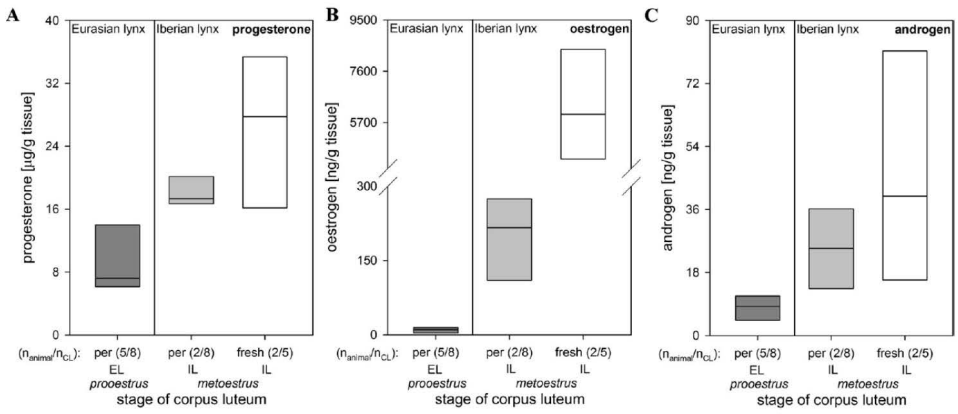

Fig. 5. Enzyme-linked immunosorbent assay analysis of the steroid content in *corpora lutea* (CLs) of *Eurasian lynxes* (prooestrus) and *Iberian lynxes* (metoestrus). Morphologically assessed stages of CLs were persistent (EL), persistent (IL) and fresh (IL). Especially the intraluteal oestrogen concentrations were substantially higher in fresh compared to perCLs of metoestrus. Steroid concentrations refer to µg/g or ng/g tissue. Data were plotted per *lynxes* and CL stage as n_{animal}/n_{CL}. EL, *Eurasian lynx*; IL, *Iberian lynx*; per, persistent.

30 times higher compared to the moderate amounts in perCLs. Moreover, depending on the sensitivity of the method of measurement, the HSD3B protein expression was almost exclusively detectable in freshCLs. In CLs of pregnant and non-pregnant domestic cats, HSD3B was shown to be the main regulatory enzyme for synthesis of active steroids as well [29,30]. In addition to the classical synthesis of P4, HSD3B also catalyses synthesis of androstenedione and testosterone as direct precursors for oestrogen synthesis [32]. Interestingly, the HSD3B pattern was most reflected in intraluteal oestrogen concentrations, which differed more than 30-fold between both CL types. As despite elevated *CYP19A1* gene expression in metoestrous perCLs the CYP19A1 protein expression was equally abundant in both types of CLs, we assume that in *Iberian lynx* CLs CYP19A1, HSD17B1 and HSD17B7 regulate the quality of biologically active oestrogens (oestrone *vs.* oestradiol), whereas precursors for oestrogen synthesis are quantitatively provided via HSD3B. In addition to HSD3B expression, the biological activity of progestogens and androgens is further regulated by steroid reductases. As described for the domestic cat CL [29], gene expression of *SRD5A1* depended on the luteal stage in the *lynx* as well, supporting its conserved role in testosterone [34] and P4 [35,36] metabolism within the feline CL.

Our findings in *Iberian lynxes* suggest that based on differences in HSD3B expression, freshCLs have a higher capacity to synthesise P4, oestrogens and androgens than perCLs. Presumably this is not restricted solely to metoestrus, as in our previous study we showed that with ongoing pregnancy, freshCLs even increase their production of P4 clearly, compared to perCLs [20]. In the present study the substantially high oestrogen concentrations in freshCLs of metoestrus coincided with the elevated expression of HSD3B. Interestingly, the enzyme expression pattern was less concordant with P4 and androgen concentrations. FreshCLs of *lynxes* were histomorphologically assigned to be in formation stage [24]. Also in domestic cats CLs of the formation stage revealed higher oestrogen concentrations compared to later stages [29]. Based on the currently available data set, we assume that HSD3B activity in freshCLs might be in favour of oestrogen synthesis, whereas with ongoing luteal phase the pathway might be switched to enhanced P4 synthesis.

The present study provides further evidence for the contribution of intraluteal steroids to constantly elevated serum steroid concentrations in *lynxes*. We earlier demonstrated, that in non-pregnant *Eurasian lynxes,* intraluteal P4 and oestrogen concentrations did not differ throughout the year [20]. In accordance, we previously measured constant serum P4 concentrations before (prooestrus) and during the mating season (oestrus) and at prolonged dioestrus in *Eurasian lynxes* [19]. Serum oestrogen concentrations remained constant throughout the annual cycle as well, except for an elevation during oestrus [19]. Similar findings were obtained for the *Iberian lynxes* in the present study, wherein serum P4 concentrations were nearly constant between pro- (Dec–Jan) and dioestrus (Apr–Nov). Our results implying the sustained synthesis of steroids by metoestrous perCLs support the earlier suggestion, that functional perCLs cause constantly elevated P4 plasma concentrations throughout the year [12,19] and, by preventing folliculogenesis and ovulation outside the breeding season, probably ensure a monooestrus cycle [12,19]. These findings might further contribute to another hypothesis on the possible role of perCLs in *lynxes*. That is, that during pregnancy CLs from previous years support freshCLs as a supplemental source of P4 to compensate luteal insufficiencies and maintain pregnancy [5,47]. This assumption is based on the observation, that the incidence of resorption or early abortion is especially high within primiparous *lynxes* [5].

The unexpectedly high serum oestrogen concentrations in *Eurasian lynxes* [19] were also shown for *Iberian lynxes*. In comparison to other felid species, serum oestrogen concentrations in *lynxes* outside the breeding season are extraordinarily high. Reference values of peak oestradiol concentrations during oestrus were, *e.g.* 59.5 ± 13.4 pg/ml in domestic cat [26], 375 pg/ml in puma [48] and 46.7 ± 6.0 pg/ml in Siberian Tiger [49], depending on the applied assay method. Although speculative, a possible luteotrophic role of high oestrogen concentrations might be considered. As discussed for rabbits and rats, oestrogen might sustain P4 secretion by the CL [50]. Moreover, the simultaneous intrauterine infusion of oestradiol and prostaglandin E_2 (PGE_2) in non-pregnant heifers demonstrated a synergism between both hormones in maintaining luteal function [51]. In an initial study we identified PGE_2 as the predominant intraluteal prostaglandin in *Eurasian lynxes*, with constantly high annual concentrations [22]. However, to support a likely luteotrophic role of both hormones to sustain functional and structural persistence of CLs in *lynxes* a more detailed study is needed. Since neither the reduced oestrogen synthesis at prooestrus nor the elevated concentrations in freshCLs were depicted in constantly high serum oestrogen concentrations, we assume a local effect of intraluteal oestrogens for both European *lynx* species. Further investigations into the origin of serum oestrogen are required to elucidate the physiological rationale for the exceptionally high oestrogen concentrations measured in *lynxes*.

Despite the limited access to *lynxes*, our study could provide an initial insight into the mechanisms of steroid biogenesis in fresh and perCLs during the particular conditions of pro- and metoestrus. The results of our study contribute further to the concept that the unique reproductive strategy of *lynxes*, which is the conversion of a poly- into a monooestrous cycle, is linked to the preservation of steroidogenic capacity in persistent CLs. Future studies should examine the annual intraluteal synthesis and reception of steroids and prostaglandins, to gain knowledge on the regulation of CL persistence. Elucidation of the reproductive strategy will strengthen attempts to increase the reproductive potential of critically endangered *Iberian lynxes*.

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Article 3: Zschockelt *et al.* 2015 *currently under review in Reproduction*

Contribution of luteal prostaglandin synthesis and reception to lifespan of feline *corpora lutea*

Contribution of luteal prostaglandin synthesis and reception to lifespan of feline *corpora lutea*

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Short title: Prostaglandins in the feline *corpus luteum*

Abbreviations

CA, *corpus albicans*; CL, *corpus luteum*; D/M, development/maintenance; EL, Eurasian lynx; ER, early regression; F, formation; freshCL, freshly formed CL; IL, Iberian lynx; LR, late regression; P4, progesterone; perCL, persistent CL; PG, prostaglandin; PGFS/AKR, prostaglandin F synthase/aldo-keto reductase; POI, postimplantation; PRI, preimplantation; PTGER, prostaglandin E receptor; PTGES, prostaglandin E synthase; PTGFR, prostaglandin F receptor; PTGS/COX, prostaglandin-endoperoxide synthase/cyclooxygenase; R, regression.

Abstract

Felids show different reproductive strategies related to the luteal phase. Domestic cats exhibit a seasonal polyoestrus and ovulation is followed by formation of *corpora lutea* (CLs). The pregnant and non-pregnant cycles are reflected by diverging plasma progesterone (P4) profiles. Eurasian and Iberian lynxes show a seasonal monooestrus, in which physiologically persistent CLs (perCLs) support constantly elevated plasma P4 levels. Prostaglandins (PGs) represent key regu-

lators of reproduction and we aimed to characterise PG synthesis pathways in feline CLs to identify their contribution to the luteal lifespan. We assessed mRNA and protein expression of PG synthases (*PTGS2/COX2*, PTGES, PGFS/AKR1C3) and PG receptors (*PTGER2*, PTGER4, PTGFR), and intraluteal levels of PGE_2 and $PGF_{2\alpha}$ in CLs of pregnant (preimplantation, postimplantation, regression stages) and non-pregnant (formation, development/maintenance, early regression, late regression stages) domestic cats, and in prooestrous Eurasian (persistent CLs, pre-mating) and metoestrous Iberian (persistent CLs, fresh CLs, post-mating) lynxes. Expression of *PTGS2/COX2*, PTGES and PTGER4 was independent of the luteal stage. High levels of luteotrophic PGE_2 in perCLs might be associated with persistence of luteal function in lynxes. Signals for PGFS/AKR1C3 expression were weak in mid and late luteal stages of cats and were absent in lynxes, concomitant with low levels of $PGF_{2\alpha}$. Thus, regulation of CL regression by luteal $PGF_{2\alpha}$ seems negligible. In contrast, expression of PTGFR was evident in nearly all investigated CLs, implying that luteal regression, *e.g.* at the end of pregnancy, is triggered by extraluteal $PGF_{2\alpha}$. Understanding the unique reproductive strategy in endangered lynxes will help to improve their reproduction potential.

1. Introduction

The reproductive biology of domestic cats (*Felis silvestris f. catus* Linnaeus, 1758) exhibits a characteristic feature: during the seasonal polyoestrus, an induced or spontaneous ovulation can be followed by either a pregnant or a non-pregnant luteal phase (Wildt *et al.* 1981, Lawler *et al.* 1993). Consequently, the formation of *corpora lutea* (CLs) and the functional luteal phase are reflected by different plasma progesterone (P4) profiles after day 10–12 *post coitum* (*p.c.*) (Paape *et al.* 1975, Verhage *et al.* 1976). Although the ongoing loss of steroidogenic capacity (Siemieniuch *et al.* 2012, Zschockelt *et al.* 2014) and the concomitant structural luteal regression (Dawson 1946, Amelkina *et al.* 2015) proceed similarly in pregnant and non-pregnant luteal phases, their CLs enter the process of luteal regression at different time points. The mechanisms regulating the prepartal and the cyclic luteal regression during the non-pregnant phase are subject to ongoing research studies (Siemieniuch *et al.* 2012, Siemieniuch *et al.* 2014, Zschockelt *et al.* 2014, Amelkina *et al.* Unpublished results).

Compared to other felid species, the reproduction of lynxes is rather unique (Göritz *et al.* 2009, Jewgenow *et al.* 2014). In contrast to the polyoestrous bobcat (*Lynx rufus* Schreber, 1777 (Parker

& Smith 1983)), the Canada (*L. canadensis* Kerr, 1792 (Fanson *et al.* 2010)), Eurasian (*L. lynx* Linnaeus, 1758 (Kvam 1991)) and Iberian lynx (*L. pardinus* Temminck, 1827 (Palomares *et al.* 2005)) are characterised by strict seasonal monooestrous strategies. The observation that central and northern European populations of both captive and free-ranging Eurasian lynxes show the same monooestrous strategy suggests that there is no plasticity regarding this phenomenon (Painer *et al.* 2014). Lynxes develop physiologically persistent CLs (perCLs, present for at least two subsequent years (Göritz *et al.* 2009, Painer *et al.* 2014)), *i.e.*, during the breeding season, freshly formed CLs (freshCLs, from a recent ovulation) coexist with previous years' perCLs with different steroidogenic capacities (Carnaby *et al.* 2012, Zschockelt *et al.* 2015) on the same ovary. The functional perCLs support constant P4 plasma levels throughout the year (Göritz *et al.* 2009, Painer *et al.* 2014) and, by preventing folliculogenesis and ovulation outside the breeding season, are hypothesised to ensure a monooestrous cycle (Göritz *et al.* 2009, Painer *et al.* 2014). Despite constant luteal activity, lynxes regularly enter oestrus and give birth at term, thus it is unclear how CLs are structurally preserved, while their function might be temporary reduced prior to oestrus and parturition.

Because there are no confounding differences between these two lynx species, regarding reproduction, the Eurasian lynx is commonly used as a model species to gain knowledge about the reproductive physiology of the Iberian lynx (Göritz *et al.* 2009, Painer *et al.* 2014, Zschockelt *et al.* 2015, Amelkina *et al.* Unpublished results). The Iberian lynx is declared as *endangered* (IUCN 2015) and the *ex situ* Iberian Lynx Conservation Breeding Programme (ILCBP (Vargas *et al.* 2008)), as well as the *in situ* Lince Andalucia – Population recovery of Iberian lynx in Andalusia Project (EU LIFE Project), were initiated to prevent the extinction of this species. Unfortunately, if mating fails, the reproductive potential of genetically valuable lynxes is consequently lost for one year, since elevated P4 prevents another cycle from occurring in the same year (Göritz *et al.* 2009, Painer *et al.* 2014). Assisted reproduction techniques, even those that are routinely used in domestic cats, when applied to captive lynxes are hindered by the persistence of CLs (Jewgenow *et al.* 2014). As a potentially helpful approach, hormone-induced luteal regression of perCLs and subsequent artificial ovulation induction could enhance the reproductive potential of lynxes (Painer *et al.* 2014). Agents used successfully to induce luteal regression in different mammalian species, among others, are prostaglandins (PGs), especially $PGF_{2\alpha}$ and its analogues (Onclin & Verstegen 1997).

The autocrine/paracrine role of luteal PGs in CL function is evident in many mammalian species (Olofsson & Leung 1994, Wiltbank & Ottobre 2003). PGs are metabolites of arachidonic acid (AA) liberated from phospholipid membranes by cytosolic phospholipase A_2 ($cPLA_2$) (Smith & Dewitt 1996). The conversion of AA into prostaglandin H_2 (PGH_2), the common precursor of PGs, is catalysed by isoforms of prostaglandin-endoperoxide synthase/cyclooxygenase (PTGS/COX) (Wiltbank & Ottobre 2003). The PGH_2 is converted to active PGs by specific synthases like prostaglandin E synthase (PTGES) for PGE_2 and several prostaglandin F synthases (PGFS; *e.g.* aldo-keto reductase family 1, member C3 (AKR1C3)) for $PGF_{2\alpha}$ (Smith & Dewitt 1996). Inactivation of PGE_2 to 13,14-dihydro-15-keto-PGE_2 (PGEM) is catalysed by HPGD (hydroxyprostaglandin dehydrogenase 15-(NAD)) (Tai *et al.* 2002). Similarly, $PGF_{2\alpha}$ is rapidly metabolised by HPGD in the lungs to its plasma metabolite 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) (Tai *et al.* 2002). PGE_2 mediates its action *via* G protein-coupled receptor subtypes, *e.g.*, PTGER2 and PTGER4. By utilising them, acting through the cAMP/PKA-pathway (Marsh & LeMaire 1974), PGE_2 was shown to stimulate P4 secretion in bovine (Kotwica *et al.* 2003), ovine (Weems *et al.* 1997) and canine (Kowalewski *et al.* 2013) luteal cell cultures, indicating a luteotrophic effect of PGE_2. In the bovine and canine CL, PGE_2 synthesis was particularly evident during the phase of luteal formation, likely being involved in CL development and growth (Arosh *et al.* 2004, Kowalewski *et al.* 2015). $PGF_{2\alpha}$ acts through its seven-transmembrane-domain receptor PTGFR which was localised in luteal cells of sheep (Juengel *et al.* 1996), pigs (Boonyaprakob *et al.* 2003), cattle (Arosh *et al.* 2004) and dogs (Kowalewski *et al.* 2008). In species in which luteal regression depends on the release of uterine $PGF_{2\alpha}$ (*e.g.* pig (Patek & Watson 1983), cattle (Kobayashi *et al.* 2002) and horse (Watson & Sertich 1990)), intraluteal production of $PGF_{2\alpha}$ was observed during the whole luteal lifespan. Thus, it was proposed that endometrial $PGF_{2\alpha}$ initiates functional luteal regression, whereas luteal $PGF_{2\alpha}$ may contribute to structural luteal regression in these species (Diaz *et al.* 2002, Hayashi *et al.* 2003).

In plasma of felids (Painer *et al.* 2014, Siemieniuch *et al.* 2014), PGFM levels increase during the last trimester of gestation, with a prepartal peak. These changes can also be mirrored non-invasively based on measurements of PGFM in urine and faecal samples (Finkenwirth *et al.* 2010, Dehnhard *et al.* 2012). In non-pregnant animals, PGFM remains at basal levels (Finkenwirth *et al.* 2010, Dehnhard *et al.* 2012). In the domestic cat, it is assumed that the pattern of plasma PGFM mainly reflects placental $PGF_{2\alpha}$ production (Tsutsui & Stabenfeldt 1993,

Siemieniuch *et al.* 2014), suggesting that $PGF_{2\alpha}$ from the pregnant uterus might contribute substantially to PGFM plasma elevation in late pregnancy. This, however, does not rule out an intraluteal source of $PGF_{2\alpha}$. In cats and lynxes, the prepartal PGFM elevation is accompanied by a decline in plasma P4 (Siemieniuch *et al.* 2012, Painer *et al.* 2014), indicative of functional luteal regression; however no structural regression, as found in the cat (Dawson 1946, Amelkina *et al.* 2015), is observed in lynxes (Painer *et al.* 2014). In addition, a smaller increase in plasma PGFM, concomitant with a P4 decrease, was shown in prooestrous lynxes (Painer *et al.* 2014), but this was not mirrored in faecal and urinary PGFM profiles (Finkenwirth *et al.* 2010, Dehnhard *et al.* 2012). Based on an earlier study on intraluteal PG synthesis in lynxes (Jewgenow *et al.* 2012), it was proposed that an intraluteal signal for the temporary functional luteal regression of perCLs might occur prior to oestrus (Jewgenow *et al.* 2014) as a prerequisite for follicular growth and ovulation.

We assume that the feline CL lifespan is regulated by the luteal capacity to synthesise and respond to PGs based on the expression of respective luteal receptors. Thus, the intraluteal expression of PG synthases (*PTGS2/COX2*, PTGES, PGFS/AKR1C3) and PG receptors (*PTGER2*, PTGER4, PTGFR), and intraluteal levels of PGE_2 and $PGF_{2\alpha}$, were assessed. With our expression studies, we aimed to deduce potential implications of intraluteal PGs (i) to initiate onset of luteal regression at the end of the pregnant and non-pregnant luteal phases in cats, (ii) to maintain CL persistence in lynxes, and (iii) to reduce the functionality of perCLs prior to oestrus. Knowledge about the mechanism of CL persistence and potential luteal regression could be implemented in conservation management plans to improve the reproductive potential of endangered Iberian lynxes.

2. Materials and Methods

2.1. Animals and tissue collection

The methods applied, and the study-design, were approved by the Internal Committee for Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research in Berlin, Germany (Permit numbers: 2010-10-01 and 2011-01-01). The Norwegian Experimental Animal Ethics Committee approved the collection of the ovarian tissue from hunted Eurasian lynxes (Permit number: 2010/161554). The tissue from Iberian lynxes was collected after ovariohysterectomy for permanent contraception at the breeding centres, which was not related to the study. The tis-

sue from domestic cats was obtained from local animal clinics after ovariohysterectomy for the purpose of permanent contraception. This was not related to the study. The tissue from late pregnancy castration of domestic cats were obtained from the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences and all procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (No. 41/2007/N and 61/2010/DTN).

Ovaries and uteri of domestic cats were collected from local animal shelters and clinics. The day of pregnancy was determined by the stage of preimplantation embryos flushed from the oviduct (< day 5 *p.c.* (Knospe 2002); a day 10 embryo was flushed from the uterine horn), the diameter of the gestation chamber (< day 20 *p.c.* (Zambelli & Prati 2006)) or the foetal crown-rump length (> day 20 *p.c.* (Schnorr & Kressin 2006)). Accordingly, CLs of pregnant cats were assigned to three stages, wherein each cat was represented by one CL: preimplantation (PRI; day 2–10, $n_{CL\ qPCR/EIA}$ = 2/4), postimplantation (POI; day 14–36, $n_{CL\ qPCR/EIA}$ = 9/6) and regression (R; day 38–48, $n_{CL\ qPCR/EIA}$ = 3/3). Because the time of ovulation was unknown and embryos or foetuses were absent, the CL stages of the non-pregnant luteal phase were determined histomorphologically according to Amelkina *et al.* (Amelkina *et al.* 2015), including parameters of cell shape, type and degree of vacuolation, nucleus condition and the ratio of non-steroidogenic to luteal cells. The CLs were assigned to four stages: formation (F; $n_{CL\ qPCR/EIA}$ = 4/6; highly neovascularised CLs contained a mixture of predominantly small transforming follicular cells with different degrees of luteinisation), development/maintenance (D/M; $n_{CL\ qPCR/EIA}$ = 11/6; polyhedral luteal cells were increased in size and exhibited accumulation of numerous lipid droplets), early regression (ER; $n_{CL\ qPCR/EIA}$ = 7/8; CLs revealed the first signs of luteal regression, large vacuoles were coarsely organised all over the cells and the ratio of non-steroidogenic to luteal cells was increased) and late regression (LR; $n_{CL\ qPCR/EIA}$ = 7/7; CLs showed extreme cell modifications with small, deformed luteal cells containing small condensed nuclei, vacuoles were without reaction to lipid staining). *Corpora albicantia* (CA), considered the last stage of the CL lifespan, were not assigned to either pregnancy or the non-pregnant luteal phase ($n_{CL\ qPCR/EIA}$ = 2/1; CAs presented highly modified luteal cells with condensed nuclei and prominently smaller luteal cells became outnumbered by non-steroidogenic cells).

Studies on CLs of Eurasian and Iberian lynxes were strictly limited by their conservation status, and collection of tissue occurred only on occasion. Fresh *post-mortem* ovarian tissue in five free-ranging Eurasian lynxes (EL1–EL5, prooestrus = pre-mating; for a detailed description of the

reproductive stages see Painer *et al.* (Painer *et al.* 2014)) was collected in February 2011 from fresh carcasses obtained from controlled, legal hunting during the national hunting quota for management purposes in Norway (Statens Naturoppsyn, SNO; Trondheim, Norway). Isolated CLs originated from previous cycle(s) and were histomorphologically staged using established domestic cat CL parameters (Amelkina *et al.* 2015, Amelkina *et al.* Unpublished results): early regression stage (EL1 $n_{CL\ qPCR} = 4$, EL2 $n_{CL\ qPCR} = 4$, EL3 $n_{CL\ qPCR} = 2$, EL4 $n_{CL\ qPCR} = 4$, EL5 $n_{CL\ qPCR} = 4$). Within the ILCBP (Portugal, Spain), two captive Iberian lynxes (IL1, IL2; day 7 *p.c.*, metoestrus = post-mating) were ovariohysterectomised in February 2013 for medical and management reasons. The CLs were isolated immediately after the surgery. The ovaries of both lynxes revealed the coexistence of perCLs (previous cycle(s), maintenance stage; IL1 $n_{CL\ qPCR} = 8$, IL2 $n_{CL\ qPCR} = 6$) and freshCLs (recent ovulation, formation stage; IL1 $n_{CL\ qPCR} = 3$, IL2 $n_{CL\ qPCR} = 5$).

Each feline CL was dissected and pieces were either fixed in Bouin's solution (for histology), or placed in RNA*later*® RNA Stabilization Reagent (for RNA isolation; Qiagen GmbH, Hilden, Germany), or transferred to Allprotect® Tissue Reagent (Eurasian lynx (SNO), hormone and protein analyses; Qiagen GmbH), or placed into liquid nitrogen (domestic cat and Iberian lynx, for hormone and protein analyses). These various solutions were applied to adequately preserve the tissues collected under the different conditions.

For hormone measurements representing the annual cycle, additional reproductive tissue (ovaries and uteri) from Eurasian lynxes was obtained from the Statens Veterinärmedicinska Anstalt (SVA; Uppsala, Sweden), where all lynx specimens harvested after legal hunting or killed by traffic accidents are delivered. The tissue was kept in frozen storage (-20 °C, in a tissue-bank facility) until processing. The isolated CLs were grouped by month and assigned to different reproductive stages according to Painer *et al.* (Painer *et al.* 2014): prooestrus (Jan, Feb), oestrus (Mar), metoestrus (Apr) and prolonged dioestrus (Sep–Dec). The respective n-numbers are depicted in Fig. 3. Ovaries of two pregnant animals obtained in April contained perCLs (markedly smaller in size, dark red in colouration) and freshCLs (bigger in size, pale colour). The pregnancies were confirmed by the presence of two foetuses in each animal.

2.2. Sequence analysis

Information on cat-specific sequences for *PTGS2/COX2* (GenBank accession EF036473 (Siemieniuch *et al.* 2011)), *PTGES* (GenBank accession GU059259 (Siemieniuch *et al.* 2013)), *PGFS/AKR1C3* (GenBank accession HM490147 (Siemieniuch *et al.* 2013)) and the validated reference gene cyclophilin A (*CYC*, GenBank accession AY029366 (Siemieniuch *et al.* 2012, Jursza *et al.* 2014)) was previously published. Based on templates of feline luteal tissue, sequence information for *PTGER2* (GenBank accession EF177829), *PTGER4* (GenBank accession EF177830) and *PTGFR* (GenBank accession AF272340) was obtained for this study (Table 1).

Total RNA isolation from reproductive tissues of Eurasian and Iberian lynxes (innuSPEED Tissue RNA/innuPREP DNase I Digest Kit; Analytik Jena AG, Jena, Germany) and reverse transcription to cDNA (RevertAid First Strand cDNA Synthesis Kit; Life Technologies GmbH, Darmstadt, Germany) were performed as previously described (Zschockelt *et al.* 2014, Zschockelt *et al.* 2015). Lynx-specific sequences of the studied genes were not annotated in GenBank prior to the study. Therefore, primers for the polymerase chain reaction (PCR) were based on gene sequences of the domestic cat or on consensus sequences derived from multiple species sequence alignments (CLC Sequence Viewer 6.7; CLC bio, Aarhus, Denmark). The primers were purchased from BioTeZ Berlin Buch GmbH (Berlin, Germany; Table 1). Partial cDNA sequences were amplified from luteal, ovarian or placental tissue using the Expand^{TM} High Fidelity^{PLUS} PCR System (Roche Diagnostics GmbH, Mannheim, Germany), as defined previously (Braun *et al.* 2012). The PCR reactions were conducted at 94 °C for 2 min followed by 35 cycles of denaturation (94 °C) for 30 sec (*PTGES*, *PGFS/AKR1C3*) or 45 sec (*PTGS2/COX2*, *PTGER2*, *PTGFR*), annealing for 30 sec (*PTGES*, *PGFS/AKR1C3*) or 45 sec (*PTGS2/COX2*, *PTGER2*, *PTGFR*; for temperatures see Table 1), elongation (72 °C) for 30 sec (*PTGES*) or 40 sec (*PGFS/AKR1C3*) or 60 sec (*PTGS2/COX2*, *PTGER2*, *PTGFR*) and a final elongation at 72 °C for 7 min. For amplification of *PTGER4* cDNA, the TrueStart^{TM} Taq DNA Polymerase Kit (Life Technologies GmbH) was used with 95 °C/2 min; 35 cycles of 95 °C/45 sec, 51 °C/45 sec, 72 °C/60 sec, and 72 °C/7 min. Purified PCR products of all genes were ligated into the pCR^{TM}4-TOPO$^{®}$ vector and transformed into One Shot$^{®}$ TOP10 cells (both from Life Technologies GmbH). Sequencing of clones or purified PCR products was performed by the Services in Molecular Biology GmbH (Dr M. Meixner, Brandenburg, Germany). Lynx-specific sequence information and validation of the reference genes β-actin (*BACT*, GenBank accession

KM458620), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, GenBank accession KM458621), ribosomal protein S7 (*RPS7*, GenBank accession JX993349) and TATA box binding protein (*TBP*, GenBank accession JX993351; all *Lynx pardinus*) were presented previously (Zschockelt *et al.* 2015).

2.3. Gene expression studies by quantitative PCR (qPCR)

2.3.1. Quantification of qPCR results using the Real-Time PCR Miner method: pregnant and non-pregnant luteal phases of domestic cat

Homogenisation of domestic cat luteal tissue (up to 22 mg), total RNA extraction and reverse transcription to cDNA was done as described above (section 2.2). No-reverse transcription controls were included to test for genomic DNA contamination. Quality and integrity of RNA were assessed using the Bioanalyzer (Agilent Technologies Deutschland GmbH, Boeblingen, Germany); RNA integrity number (RIN) values were above 7.0. Additionally, RNA concentration and purity were validated by the NanoDrop ND-1000 (Peqlab Biotechnologie GmbH, Erlangen, Germany). The relative mRNA expression of target genes was determined by qPCR. The primers were either chosen based on previous studies (*PTGS2/COX2* (Siemieniuch *et al.* 2011), *PTGES* (Siemieniuch *et al.* 2013), *PGFS/AKR1C3* (Siemieniuch *et al.* 2013)) or designed with the free online primer design tool Primer3 (http://simgene.com; *PTGER2*, *PTGER4*, *PTGFR*). All primers were purchased from BioTez Berlin Buch GmbH (Table 1). The qPCR reactions were carried out in an automated fluorometer ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) using the SYBR® Green PCR Master Mix (Applied Biosystems). The total qPCR reaction volume was 10 µl containing 3 µl cDNA (corresponding to 10 ng of total RNA for genes of interest and the reference gene), 1 µl each of forward and reverse primers (500 nM), and 5 µl SYBR Green PCR Master Mix + 0.1 µl CRX. The qPCR conditions were as follows: initial denaturation (10 min at 95 °C), followed by 40 cycles of denaturation (15 sec at 95 °C), and annealing/elongation (60 sec at 60 °C). After each qPCR reaction, melting curves were obtained by stepwise increases in temperature from 60 to 95 °C to ensure single product amplification. Among several different genes, *CYC* was selected as reference gene (Siemieniuch *et al.* 2012, Jursza *et al.* 2014). Quantification of qPCR results was performed by normalising the signals of target genes with the *CYC* signal using the Real-Time PCR Miner method, whereby calculation of reaction efficiency and the fractional cycle number at threshold (CT) were based on the kinetics of individual qPCR reactions (Zhao & Fernald 2005).

2.3.2. *Quantification of qPCR results using a standard curve: prooestrus and metoestrus of Eurasian and Iberian lynx*

Lynx luteal tissue (14–26 mg) was homogenised, total RNA was extracted and reverse transcribed to cDNA as described above (section 2.2). No-reverse transcription controls were included and RNA concentration and purity were determined; RIN values were above 6.1. Intron-spanning primers for qPCR were designed according to sequences identified in the present study (Table 1). For qPCR, diluted cDNA (corresponding to 2 or 10 ng of total RNA for genes of interest; 4 ng for reference genes) was analysed with the CFX96® Real-Time PCR Detection System using the SsoFast™ EvaGreen® Supermix (both from Bio-Rad Laboratories GmbH, Munich, Germany). Conditions for qPCR were: 98 °C for 2 min and 40 cycles of denaturation at 98 °C for 8 sec and annealing/elongation for 8 sec at different temperatures (Table 1). The qPCR products were quantified with the CFX Manager™ Software 1.6 (Bio-Rad Laboratories GmbH). Because cloned cDNA of lynx-specific genes was generated for sequence analysis (see section 2.2.), standard dilutions for calibration of qPCR results were derived from the respective external recombinant plasmid DNA. Fragments of *BACT*, *GAPDH*, *RPS7* and *TBP* were amplified for normalisation as described previously (Zschockelt *et al.* 2015). A multiple normalisation factor based on the reference gene expression was calculated for individual CLs referring to Vandesompele *et al.* (Vandesompele *et al.* 2002).

2.4. *Protein expression studies by immunohistochemistry*

For immunohistochemical analysis, 3 µm sections of Bouin-fixed and paraffin-embedded CLs were mounted on microscope slides (Superfrost™ Plus; Thermo Fisher Scientific, Braunschweig, Germany). The immunohistochemical procedure of the immunoperoxidase method for PTGES, PTGER4 and PGFS/AKR1C3 was described previously (Gram *et al.* 2013, Gram *et al.* 2014). For PTGFR, a protocol referring to a previous study (Braun *et al.* 2012) was applied. Briefly, slides were deparaffinised in xylol (for PTGFR in Roti®-Histol; Carl Roth GmbH, Karlsruhe, Germany) and rehydrated in ethanol. Antigen retrieval was performed in citrate buffer (10 mM, pH 6.0). Endogenous peroxidase activity was quenched by incubation in 0.3 % (for PTGFR in 3 %) H_2O_2 with methanol. Afterwards, the slides were incubated in 10 % goat serum (for PTGFR in 5 % BSA in PBS) in order to block non-specific binding sites. Thereafter, the slides were overlaid overnight with canine-specific polyclonal antibodies: guinea pig anti-PTGES (1:2500 (Gram

et al. 2014, Kautz *et al.* 2014)), anti-PGFS/AKR1C3 (1:750 (Gram *et al.* 2013); both custom made by Eurogentec, Seraing, Belgium) and rabbit anti-PTGER4 (1:750 (Gram *et al.* 2014); 101775, Cayman Chemical Company, Ann Arbor, MI, USA). For the isotype control, slides were incubated with pre-immune guinea pig serum (PTGES, PGFS/AKR1C3) or rabbit IgG irrelevant antibodies I-1000 (PTGER4; all from Vector Laboratories, Inc., Burlingame, CA, USA) at the same dilution as the primary antibodies. The slides were subsequently incubated with biotinylated secondary goat anti-guinea pig IgG BA-7000 antibodies at 1:100 dilution (PTGES, PGFS/AKR1C3) or goat anti-rabbit IgG BA-1000 at 1:100 dilution (PTGER4; all from Vector Laboratories). The signals for PTGES, PTGER4 and PGFS/AKR1C3 were enhanced with the streptavidin-avidin-peroxidase Vectastain® ABC kit (Vector Laboratories). The PTGFR antibody was used at a 1:100 dilution (PGF2αR (H-55), sc-67029; Santa Cruz Biotechnology, Inc., Heidelberg, Germany). For the negative control, PTGFR slides were incubated with blocking solution instead of primary antibody to test for specificity of staining. Slides for PTGFR analysis were subsequently incubated with goat anti-rabbit IgG-HRP (K4002; Dako Deutschland GmbH, Hamburg, Germany) as secondary antibody. For all slides, the peroxidase activity was finally detected using the Liquid DAB Substrate Kit (Dako North America, Inc., CA, USA). Sections were counterstained with haematoxylin, dehydrated in ethanol and covered with Histokitt (Assistant, Osterode, Germany) or Roti®-Histokitt (for PTGFR; Carl Roth GmbH) and cover-slips. Slides were analysed with an Axioplan microscope combined with a ProgRes® C10^{plus} camera (both Carl Zeiss MicroImaging GmbH, Goettingen, Germany) and the Cell^P Soft Imaging Software (Olympus Soft Imaging Solutions GmbH, Muenster, Germany).

2.5. Intraluteal hormone studies by Enzyme-Linked Immunosorbent Assay

The homogenisation of luteal tissue and the subsequent hormone extraction was done as previously described for domestic cat (Zschockelt *et al.* 2014, Amelkina *et al.* 2015) and lynx (Carnaby *et al.* 2012, Zschockelt *et al.* 2015) CLs. Briefly, aliquots of luteal homogenates (8–68 mg luteal tissue for cats, 9–65 mg luteal tissue for lynxes) were twice extracted with tert-butyl methyl ether (TBME)/petroleum ether (PE) and extracts were eventually dissolved in 40 % methanol. PGE_2 analyses were carried out using the commercial DetectX® Prostaglandin E_2 Enzyme Immunoassay Kit (Ann Arbor) according to the manufacturer's protocol. The stated cross-reactivity was as follows: 100 % to PGE_2, 2 % to $PGF_{2\alpha}$, < 0.3 % to 6-keto- $PGF_{2\alpha}$ and < 0.1 % to PGFM. Intra- and inter-assay coefficients of variation (CVs) for three biological samples were

7.4 % (n = 20) and 10.4 % (n = 15) respectively. The determination of intraluteal $PGF_{2\alpha}$ concentrations was carried out in the same extracts, using an in-house $PGF_{2\alpha}$ Enzyme-Linked Immunosorbent Assay (EIA) as described earlier (Dehnhard & Jewgenow 2013). The $PGF_{2\alpha}$ antibody was raised in rabbits immunised against $PGF_{2\alpha}$-BSA and was described to cross-react to 34 % with $PGF_{1\alpha}$ and to less than 0.01 % with PGE_2, PGFM and PGEM (Claus *et al.* 1990). Intra- and inter-assay CVs for two biological samples were 4.6 % (n = 10) and 7.1 % (n = 10).

2.6. Statistical analysis

Due to animal health issues and difficulties in obtaining larger amounts of samples, CLs of pregnant domestic cats and endangered Eurasian and Iberian lynxes were obtained randomly and were rarely available for the study. Thus, the number of replicates was limited and statistical analysis was only possible to some extent. The R software package (R: A language and environment for statistical computing, version 2.15.2, Vienna, Austria) was applied for statistical analysis (values of $p \leq 0.05$ were considered significant). For the non-pregnant luteal phase of domestic cats, the non-parametric Kruskal-Wallis rank sum test was applied to determine the influence of luteal stage on the relative mRNA expression and hormone levels. The Wilcoxon rank sum test was used for *post-hoc* pairwise comparison (*p*-value adjustment: Benjamini-Hochberg).

For the two Iberian lynxes, the non-parametric Mann-Whitney U-test was applied to determine the influence of persistent and fresh stages of CLs on the relative amount of mRNA copies per individual lynx. Other than this, results were analysed descriptively and the distribution of mRNA expression (n_{CL}) and intraluteal prostaglandin levels (n_{CL} or n_{animal}/n_{CL}) were depicted in vertical Box Plots by plotting data as median and percentiles. The PGE_2:$PGF_{2\alpha}$ ratio was depicted in vertical bar charts with simple error bars representing standard deviations (n_{CL} or n_{animal}/n_{CL}; SigmaPlot® 10.0, Systat Software GmbH, Erkrath, Germany). The mRNA expression of *PTGS2/COX2* and *PTGER2* was presented in tabular fashion as single values or mean values ± standard deviation (n_{CL}).

3. Results

3.1. Intraluteal mRNA expression of PGE_2 and $PGF_{2\alpha}$ synthases and receptors (PTGS2/COX2, PTGES, PTGER2, PTGER4, PGFS/AKR1C3, PTGFR)

Because no statistical analysis could be performed for the pregnant luteal phase of the domestic cat, the qPCR analyses only in tendency revealed that the intraluteal gene expression of *PTGS2/COX2* (Table 2), *PTGES* (Fig. 1A), *PTGER2* (Table 2) and *PGFS/AKR1C3* (Fig. 1C) appeared to be higher in the preimplantation stage compared to the postimplantation and regression stages. In contrast, for *PTGER4* (Fig. 1B) and *PTGFR* (Fig. 1D) the relative mRNA expression tended to be higher in the regression stage. During the non-pregnant luteal phase of the cat, the expression of *PTGS2/COX2* (Table 2), *PTGES* (Fig. 1A), *PTGER2* (Table 2) and *PGFS/AKR1C3* (Fig. 1C) remained unchanged ($p_{NP} > 0.05$), whereas expression of *PTGER4* ($p_{NP} = 0.05$; Fig. 1B) and *PTGFR* ($p_{NP} = 0.03$; Fig. 1D) was affected by the luteal stage. In detail, the expression of *PTGER4* was lower in the development/maintenance stage compared to the late regression stage ($p_{NP} = 0.035$). For *PTGFR*, the expression was higher in the development/maintenance stage ($p_{NP} = 0.018$), early regression stage ($p_{NP} = 0.048$) and late regression stage ($p_{NP} = 0.018$) compared to the formation stage. *Corpora albicantia* exhibited notably high relative mRNA expression for *PTGER4* (Fig. 1B) and *PGFS/AKR1C3* (Fig. 1C).

For Iberian lynxes, the relative amount of mRNA copies was independent of the luteal stage for *PTGS2/COX2* (Table 3), *PTGES* (Fig. 2A) and *PTGER4* (Fig. 2B; for all $p_{IL1/IL2} > 0.05$). The amount of mRNA for *PTGER2* (Table 3) and *PGFS/AKR1C3* (Fig. 2C) was higher in metoestrous perCLs compared to freshCLs in one of the two animals (for both genes: $p_{IL1} = 0.0121$; $p_{IL2} > 0.05$). Both animals revealed higher mRNA amounts of *PTGFR* (Fig. 2D) in perCLs compared to freshCLs ($p_{IL1} = 0.0121$, $p_{IL2} = 0.0043$).

Medians of relative mRNA amounts in perCLs of prooestrous Eurasian lynxes (SNO, Norway; no statistical analysis) tended to be equal (*PTGES*, Fig. 2A*; PGFS/AKR1C3* Fig. 2C) or intermediate (*PTGFR*; Fig. 2D) compared to perCLs and freshCLs of metoestrous Iberian lynxes. For *PTGS2/COX2* (Table 3), *PTGER2* (Table 3) and *PTGER4* (Fig. 2B) the medians tended to be higher in perCLs of Eurasian lynxes.

3.2. Cellular localisation and protein expression of PGE_2 and $PGF_{2\alpha}$ synthases and receptors (PTGES, PTGER4, PGFS/AKR1C3, PTGFR)

As revealed by immunohistochemistry, staining for protein expression of PG synthases and receptors was localised to luteal cells. During the pregnant (data not shown) and the non-pregnant luteal phases of the domestic cat, no changes were observed in intensity of the luteal signal for PTGES (Fig. 1E) and PTGER4 (Fig. 1F). Also, the *corpus albicans* exhibited prominent staining (Fig. 1E+F). The protein expression of PGFS/AKR1C3 remained constantly low at all time points investigated during pregnancy (data not shown). In non-pregnant cats (Fig. 1G), no signals for PGFS/AKR1C3 protein expression were found in the formation stage, but weak staining was detected during the development/maintenance stage. The signals were then slightly more intense during the early regression stage and the late regression stage. In the *corpus albicans* no signal was detected. The expression of PTGFR was equally strong at days 4, 24 and 48 *p.c.* in CLs of pregnant cats (data not shown). During the non-pregnant phase, expression of PTGFR was weak during the early luteal phase (Fig. 1H), absent in the development/maintenance stage, but clearly evident in the early regression stage, the late regression stage and the *corpus albicans*.

At prooestrus and metoestrus of Eurasian (SNO, Norway) and Iberian lynxes, the observed intensity of the luteal signal for PTGES (Fig. 2E) and PTGER4 (Fig. 2F) was equally strong between perCLs and freshCLs. In all analysed lynx CLs no signals for PGFS/AKR1C3 protein expression were present (Fig. 2G), but staining for PTGFR was evident (Fig. 2H). The dot-shaped staining detected in perCLs of both lynxes stained with different antibodies were considered non-specific, as they were found in control slides as well.

3.3. Intraluteal PGE_2 and $PGF_{2\alpha}$ concentrations

By using EIA, it was revealed that the PGE_2 level measured in CLs of pregnant domestic cats (Fig. 3A) tended to decrease from the preimplantation stage to the regression stage (no statistical analysis). During the non-pregnant luteal phase (Fig. 3A), the PGE_2 level changed ($p_{NP} = 0.038$), but the *post-hoc* test failed to reveal differences between specific groups. In the single *corpus albicans* sample, a moderate PGE_2 concentration was measured (196.55 ng/g). The concentration of $PGF_{2\alpha}$ tended to be numerically higher in the postimplantation stage and regression stage compared to the preimplantation stage of pregnancy (Fig. 3B). For non-pregnant cats, the intraluteal $PGF_{2\alpha}$ level was unaffected by the luteal stage ($p_{NP} > 0.05$; Fig. 3B). Relatively high $PGF_{2\alpha}$ levels

were noted for the single *corpus albicans* available for the study (22.17 ng/g). In general, the maximum levels of PGE_2 (1845.50 ng/g, formation stage) were considerably higher compared to $PGF_{2\alpha}$ (22.17 ng/g, *corpus albicans*). The ratio of PGE_2:$PGF_{2\alpha}$ appeared to be in favour of PGE_2 especially at preimplantation, but was constant throughout the non-pregnant luteal phase ($p_{NP} > 0.05$; Fig. 3E).

During the annual cycle of Eurasian lynxes (SVA, Sweden; Fig. 3C, no statistical analysis), the median levels of intraluteal PGE_2 tended to be high in January (5334 ng/g; prooestrus) and April (4866.20 ng/g; metoestrus). Low median levels were measured in December (1666.40 ng/g; prolonged dioestrus). The CLs obtained in April (metoestrus) from one pregnant Eurasian lynx could be divided into perCLs and freshCLs. The median PGE_2 level appeared to be numerically higher in perCLs (5005.10 ng/g) than in freshCLs (2214 ng/g, no statistical analysis; Fig. 4A). The intraluteal level of $PGF_{2\alpha}$ seemed to be almost constant throughout the year (Fig. 3D). During April (metoestrus), $PGF_{2\alpha}$ values were similar between the two investigated pregnant Eurasian lynxes and a single non-pregnant female. For the pregnant lynxes, the median $PGF_{2\alpha}$ concentration tended to be higher in perCLs (298.19 ng/g) compared to freshCLs (34.88 ng/g; Fig. 4B). In Eurasian lynxes the maximum levels of PGE_2 were substantially higher (9440.60 ng/g; January) compared to intraluteal $PGF_{2\alpha}$ levels (2206.30 ng/g; April) and the PGE_2:$PGF_{2\alpha}$ ratio revealed prevalence towards PGE_2, with a tendency towards higher ratios in January (prooestrus), March (oestrus) and December (prolonged dioestrus; Fig. 3F).

4. Discussion

This study presents a detailed analysis of intraluteal PGE_2 and $PGF_{2\alpha}$ synthesis and reception in relation to the different reproductive strategies of the domestic cat and the two European lynx species. We demonstrated that the feline CL is capable of synthesising PGs *de novo* during the pregnant and non-pregnant luteal phases in cats and at prooestrus and metoestrus of lynxes. While there is a distinct prevalence towards intraluteal synthesis of PGE_2 rather than $PGF_{2\alpha}$, a potential receptivity of the feline CL to intraluteal and extraluteal PGE_2 and $PGF_{2\alpha}$ signals is evident.

Luteal cells are able to convert arachidonic acid to PGH_2, whereby isoforms of PTGS/COX differ in the regulation of expression and tissue distribution (Wiltbank & Ottobre 2003). The luteal expression of PTGS2/COX2 is up-regulated at the beginning of the CL phase in dogs

(Kowalewski *et al.* 2006, Kowalewski *et al.* 2015). Likewise, luteal concentrations of *PTGS2/COX2* mRNA are greater in the early compared to mid and late luteal phases in the cow (Kobayashi *et al.* 2002). In contrast, the protein content of PTGS2/COX2 is high in the mouse ovary during CL regression (Sander *et al.* 2008) and the PTGS/COX activity increases from the early to mid and late luteal phases in human CLs (Mitchell *et al.* 1991). The variations in the time course of intraluteal PG production among species might be related to differences in luteolytic signalling during CL regression and the autocatalytic effect of uterine $PGF_{2\alpha}$. Small amounts of uterine $PGF_{2\alpha}$ dramatically amplify intraluteal $PGF_{2\alpha}$ production (and PTGS2/COX2 expression) in species with uterine-dependent luteal regression (Diaz *et al.* 2002), such as pig (Patek & Watson 1983), cattle (Kobayashi *et al.* 2002) and horse (Watson & Sertich 1990). Our results demonstrated the steady-state expression of *PTGS2/COX2* mRNA in the feline CL. Since survival of the cat CL is presumably dependent on the presence of external luteotrophic signals (Tsutsui & Stabenfeldt 1993), an increase of *PTGS2/COX2* expression towards the end of the CL lifespan was suggested. It might be hypothesised that due to a possible lack of luteotrophic support during the non-pregnant luteal phase, serum P4 drops from day 21 onwards (Verhage *et al.* 1976), accompanied by a gradual morphological degradation of the CL (Dawson 1941, Amelkina *et al.* 2015). This expected change was not mirrored by any shift in *PTGS2/COX2* mRNA expression. Moreover, the freshCLs collected from the two Iberian lynxes on day 7 *p.c.* did not differ in the expression of *PTGS2/COX2* compared to perCLs of the same ovary.

Our study confirmed the presence of *PTGES* and *PGFS/AKR1C3* mRNA, as well as PGE_2 and $PGF_{2\alpha}$, in feline CL tissue. Because the coexistence of potentially luteotrophic PGE_2 and luteolytic $PGF_{2\alpha}$ suggests a system of equilibrium, the ratio of PGE_2 and $PGF_{2\alpha}$ throughout the luteal phase seems to be of major functional relevance. Although no statistically significant changes in the course of *PTGES* and *PGFS/AKR1C3* mRNA expression were found in the cat, a potential shift from high PGE_2:$PGF_{2\alpha}$ ratios during the early luteal phase towards decreased ratios in later CL stages might be concluded from the available data set. A PGE_2:$PGF_{2\alpha}$ equilibrium shift is characteristic for different stages of luteal development in other species as well. In mice, for example, ovarian tissue showed increased $PGF_{2\alpha}$ levels at the expense of PGE_2 during luteal regression (Sander *et al.* 2008).

Similar to other species (Vijayakumar & Walters 1983, Olofsson *et al.* 1992, Miller & Pawlak 1994), PGE_2 was the predominant PG in feline luteal tissue, with high concentrations throughout

the pregnant and non-pregnant luteal phases of the cat and during the annual cycle of Eurasian lynxes. Our remarkable finding that perCLs contained higher intraluteal PGE_2 levels than freshCLs in lynxes, might bring forth a new hypothesis on the luteotrophic role of PGE_2 in the persistence of CLs. The maintained steroidogenic activity of perCLs outside the breeding season in Eurasian (Carnaby *et al.* 2012, Painer *et al.* 2014) and Iberian lynxes (Zschockelt *et al.* 2015), is most likely associated with elevated serum P4 levels throughout the year, together with extraordinarily high serum oestrogen levels compared to other felid species (Schmidt *et al.* 1979, Goodrowe *et al.* 1988, Swanson *et al.* 1996). As discussed for rabbits and rats, oestrogen might sustain P4 secretion by the CL (Keyes *et al.* 1979). Moreover, the simultaneous intrauterine infusion of oestradiol and PGE_2 in non-pregnant heifers demonstrated a synergism between these hormones in maintaining luteal function (Reynolds *et al.* 1983). The luteotrophic action of PGE_2 (and oestradiol) might support permanent P4 production from perCLs outside the breeding season, which is presumed to ensure a monooestrous cycle (Göritz *et al.* 2009, Painer *et al.* 2014). In addition to our previous hypothesis on perCLs as a supplemental source of P4 (Zschockelt *et al.* 2015), our present findings on high intraluteal PGE_2 synthesis might propose the luteotrophic support of freshCLs by CLs from previous years (Woshner *et al.* 2001, Jewgenow *et al.* 2014).

For PGE_2, several receptors have been described, with PTGER2 and PTGER4 being relevant for protecting the CL from luteolytic challenges during early pregnancy in sheep (Lee *et al.* 2012), and PTGER2 being responsible for stimulation of P4 production *via* regulation of luteal 11betaHSD1 activity in humans (Chandras *et al.* 2007). In cattle (Arosh *et al.* 2004) and dioestrous dogs (Kowalewski *et al.* 2008), *PTGER2* mRNA exhibited highest expression in the growing CL, whereas in pregnant dogs, PTGER2 declined at *pre-partum* (Kowalewski *et al.* 2013). PTGER4 showed a constant expression pattern during the canine luteal lifespan (Kowalewski *et al.* 2008, Kowalewski *et al.* 2013). The results of our study imply that reception of PGE_2 in the feline CL is mainly independent of the luteal and reproductive stages. The confirmed high expression of PGE_2 receptors in CLs of lynxes, together with high PGE_2 and oestradiol contents, might be one of the crucial intraluteal mechanisms fulfilling the luteotrophic requirements for functional and structural maintenance of perCLs.

The capacity of the feline CL to provide $PGF_{2\alpha}$ was limited and in agreement with low mRNA expression of *PGFS/AKR1C3*; only weak protein signals for PGFS/AKR1C3 were detected at some CL stages of cats but no signals were found in CLs of lynxes. During pregnancy and dioe-

strus in dogs, only weak or no intraluteal PGFS/AKR1C3 expression was detectable as well (Kowalewski *et al.* 2008, Gram *et al.* 2013). We conclude that the local luteal production of $PGF_{2\alpha}$ is presumably not involved in the initiation of the onset of prepartal and cyclic luteal regression in felids. For pre-term felids, the feline placenta appears to be one of the main sources of luteolytic $PGF_{2\alpha}$, because placental $PGF_{2\alpha}$ and its plasma metabolite PGFM were elevated during the last trimester of pregnancy (Siemieniuch *et al.* 2014). This is different than the situation in dogs, in which an immediate prepartal increase of placental $PGF_{2\alpha}$ occurs only 12–48h prior to parturition (Kowalewski *et al.* 2010). In lynxes CLs persist for at least two years (Painer *et al.* 2014) and retain their structural integrity (Jewgenow *et al.* 2012, Amelkina *et al.* Unpublished results). Thus, the general assumption that functional luteal regression, as initiated by extraluteal $PGF_{2\alpha}$, precedes structural luteal regression by intraluteal amplification of the luteolytic signal (Diaz *et al.* 2002, Hayashi *et al.* 2003), might not fully account for perCLs in lynxes, in which absence of an internal $PGF_{2\alpha}$ signal might prevent final initiation of the CL demise.

Our results imply that the capacity of feline CLs to respond to $PGF_{2\alpha}$ *via* expression of PTGFR is already acquired after CL formation but is mainly independent of the luteal stage thereafter. A change in receptivity towards $PGF_{2\alpha}$ was observed only for non-pregnant cats, in which clear signals of PTGFR protein expression were first evident from early regression onwards. Similarly, *PTGFR* mRNA levels increased from the early to the mid luteal phase in the cow (Arosh *et al.* 2004), rabbit (Boiti *et al.* 2001), pig (Boonyaprakob *et al.* 2003) and dog (Kowalewski *et al.* 2008, Kowalewski *et al.* 2009). The steady-state expression of PTGFR on days 4, 24 and 48 of pregnancy does not explain studies on domestic cats, in which abortion induction with $PGF_{2\alpha}$ was not possible before day 33 (Wildt *et al.* 1979, Verstegen *et al.* 1993) or even day 40 (Nachreiner & Marple 1974) of gestation. However, the high expression of PTGFR at day 48 might be a prerequisite for the CL to respond to elevated PGFM levels during the last trimester of pregnancy in cats (Finkenwirth *et al.* 2010).

In prooestrous lynxes, expression of PTGFR was present in luteal cells of perCLs. If the prooestrous expression potentially reflects a constant intraluteal expression of PTGFR, as likewise shown for the cat (this study), it might explain why, after treatment of female lynxes with cloprostenol (a $PGF_{2\alpha}$ analogue; outside the breeding season) (Painer *et al.* 2014), the ovarian blood supply was acutely reduced, accompanied by a drop in serum P4 to baseline. As the blood supply and steroid production were reconstituted (Painer *et al.* 2014), we hypothesise that $PGF_{2\alpha}$-

induced vasoconstriction, and a subsequent reduction of luteal blood flow, may trigger a functional but reversible shutdown of steroid release from the CL by, *i.a.*, depriving substrates for steroid biogenesis (Pharriss *et al.* 1970). It seems, however, contradictory, that during metoestrus, perCLs retain their structural integrity while still expressing receptors for potentially luteolytic $PGF_{2\alpha}$. It might be hypothesised that next to the lack of a substantial endogenous luteolytic $PGF_{2\alpha}$ signal, the preservation of the CL structure is further caused by an interference of anti-apoptotic factors. Despite expression of PTGFR, and possible initiation of the luteolytic signal, the signal transduction *via* the apoptosis cascade (Amelkina *et al.* Unpublished results) might be prevented.

In conclusion, our data implicate a potential role of PGE_2 in maintaining CL persistence in lynxes, thus ensuring their atypical ovarian cycle compared to other felids. A temporal functional luteal regression might therefore be triggered by ligand activation of PTGFR, depending on extraluteal signals. Future studies should focus on potential factors involved in the induction of a transitory luteal vasoconstriction and the concurrent prevention of structural CL demise. Understanding the mechanisms of physiological CL persistence will support the establishment of protocols for assisted reproduction to improve the reproduction potential of endangered lynx species.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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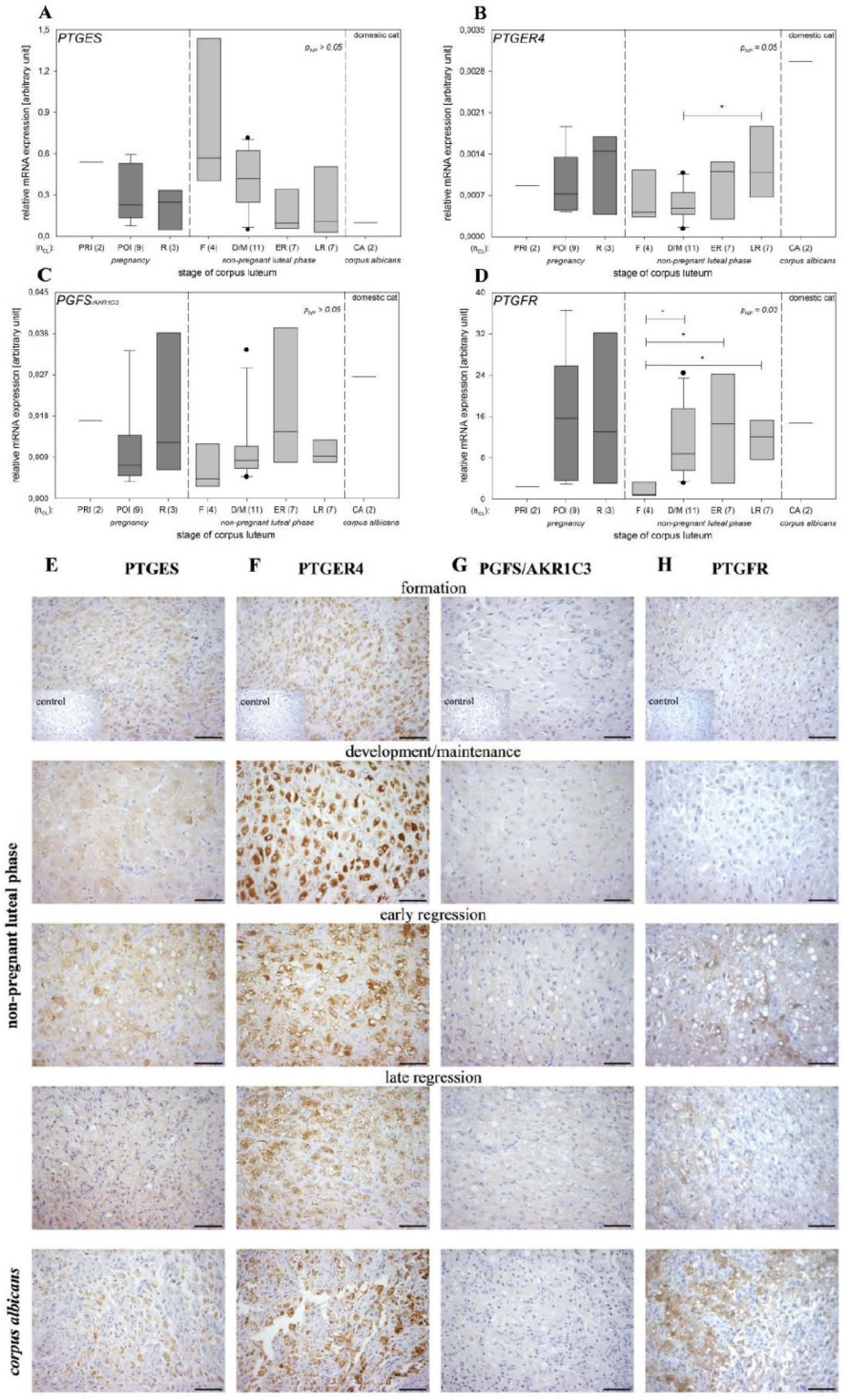
A
PTGES
relative mRNA expression [arbitrary unit]
domestic cat
PRI (2)
POI (9)
R (3)
F (4)
DM (11)
ER (7)
LR (7)
CA (2)
pregnancy
non-pregnant luteal phase
corpus albicans
stage of corpus luteum
B
PTGER4
C
PGFS/AKR1C3
D
PTGFR
E PTGES
F PTGER4
G PGFS/AKR1C3
H PTGFR
formation
development/maintenance
early regression
late regression
non-pregnant luteal phase
corpus albicans
control

Fig. 1

Figure 1. Intraluteal mRNA and protein expression of PGE_2 and $PGF_{2\alpha}$ synthases and receptors during the pregnant and non-pregnant luteal phases and in *corpora albicantia* of the domestic cat. The *PTGES* (A), *PTGER4* (B), *PGFS/AKR1C3* (C) and *PTGFR* (D) relative mRNA expression [arbitrary unit] was determined by quantitative PCR (qPCR; Real-Time PCR Miner method), normalised with a factor calculated from qPCR results of a reference gene. The Kruskal-Wallis rank sum test was applied for the non-pregnant luteal phase; asterisks indicate statistical differences ($p_{NP} \leq 0.05$). Data were plotted per CL stage as n_{CL} (one CL per cat). Protein expression of PTGES (E), PTGER4 (F), PGFS/AKR1C3 (G) and PTGFR (H) was analysed for the non-pregnant luteal phase and in the *corpus albicans* by immunohistochemistry. Insets show isotype (PTGES, PTGER4, PGFS/AKR1C3) and negative controls (PTGFR). Scale bars represent 50 µm. CA, *corpus albicans*; CL, *corpus luteum*; D/M, development/maintenance; ER, early regression; F, formation; LR, late regression; NP; non-pregnant luteal phase; POI, postimplantation; PRI, preimplantation; R, regression.

Figure 2. Intraluteal mRNA and protein expression of PGE_2 and $PGF_{2\alpha}$ synthases and receptors in Eurasian (prooestrus) and Iberian (metoestrus) lynx. The *PTGES* (A), *PTGER4* (B), *PGFS/AKR1C3* (C) and *PTGFR* (D) relative amount of mRNA copies [per ng total RNA] are copy numbers determined by a calibration curve in quantitative PCR (qPCR) that were afterwards normalised with a factor calculated from qPCR results of reference genes. Values refer to 1 ng original total RNA. The Mann-Whitney U-test was applied for the two Iberian lynxes; asterisks indicate statistical differences ($p_{IL1/IL2} \leq 0.05$). Data were plotted per individual lynx and CL stage as n_{CL}. Protein expression of PTGES (E), PTGER4 (F), PGFS/AKR1C3 (G) and PTGFR (H) was analysed for perCLs and freshCLs of prooestrus and metoestrus by immunohistochemistry. Insets show isotype (PTGES, PTGER4, PGFS/AKR1C3) and negative controls (PTGFR). Scale bars represent 50 µm. CL, *corpus luteum*; EL, Eurasian lynx; fresh, freshly formed CL; IL, Iberian lynx; per, persistent CL.

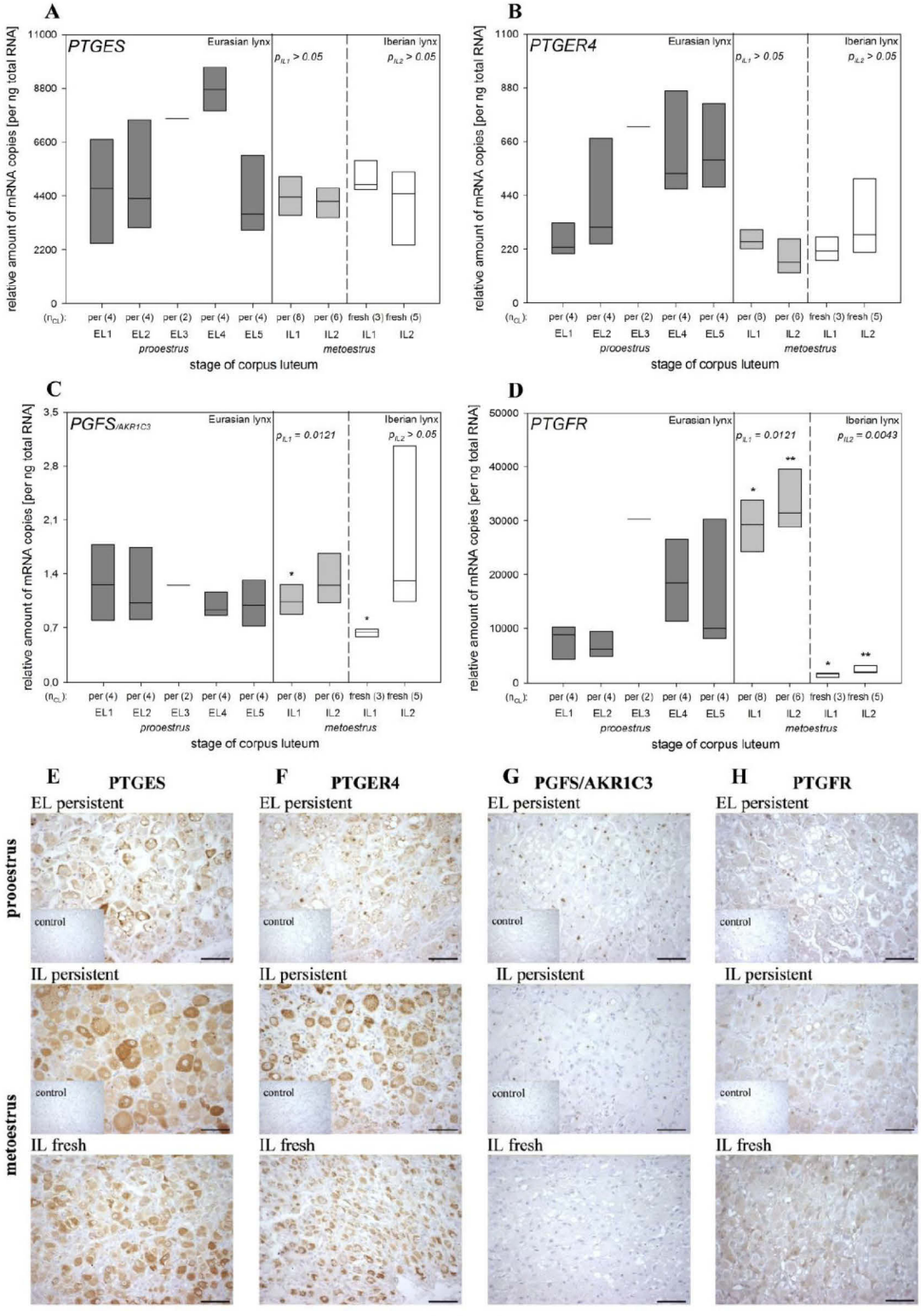
A
PTGES
Eurasian lynx
Iberian lynx
relative amount of mRNA copies [per ng total RNA]
stage of corpus luteum
prooestrus
metoestrus
B
PTGER4
C
PGFS/AKR1C3
D
PTGFR
E PTGES
F PTGER4
G PGFS/AKR1C3
H PTGFR
EL persistent
IL persistent
IL fresh
control
prooestrus
metoestrus

Fig. 2

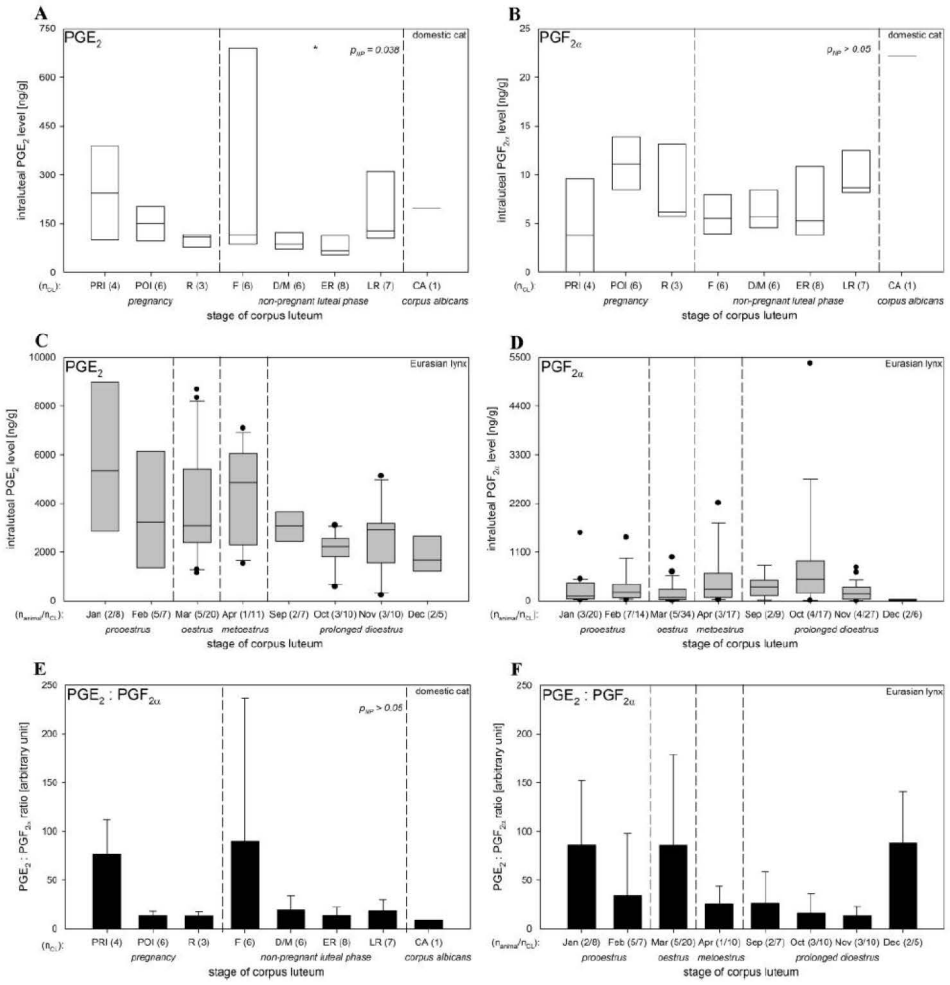

Fig. 3

Figure 3. Intraluteal levels of PGE_2 and $PGF_{2\alpha}$ during the pregnant and non-pregnant luteal phases and in *corpora albicantia* of the domestic cat and during the annual cycle of the Eurasian lynx. The PGE_2 (A+C) and $PGF_{2\alpha}$ (B+D) levels [ng/g tissue] were determined by Enzyme-Linked Immunosorbent Assay. The PGE_2:$PGF_{2\alpha}$ ratio [arbitrary unit] was calculated (E+F) by division of PGE_2 values by $PGF_{2\alpha}$ values. The Kruskal-Wallis rank sum test was applied for the non-pregnant luteal phase; asterisks indicate statistical differences ($p_{NP} \leq 0.05$). Data were plotted per CL stage as n_{CL} (one CL per cat) or per lynxes and month as n_{animal}/n_{CL}. CA, *corpus albicans*; CL, *corpus luteum*; D/M, development/maintenance; ER, early regression; F, formation; LR, late regression; NP, non-pregnant luteal phase; POI, postimplantation; PRI, preimplantation; R, regression.

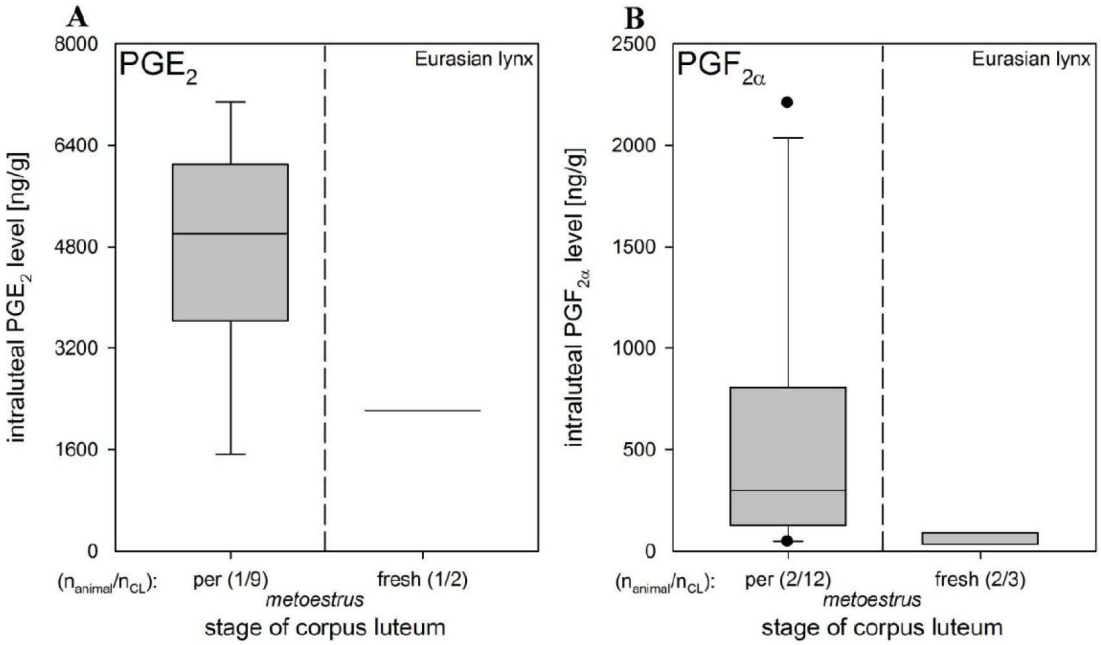

Fig. 4

Figure 4. Intraluteal levels of PGE_2 and $PGF_{2\alpha}$ in persistent and fresh *corpora lutea* of metoestrous Eurasian lynx. The PGE_2 (A) and $PGF_{2\alpha}$ (B) levels [ng/g tissue] were determined by Enzyme-Linked Immunosorbent Assay. Ovaries of two pregnant animals obtained in April (metoestrus; see also Fig. 3) contained perCLs (markedly smaller in size, dark red in colouration) and freshCLs (bigger in size, pale colour) that were analysed separately. Data were plotted per lynx and CL stage as n_{animal}/n_{CL}. CL, *corpus luteum*; fresh, freshly formed CL; per, persistent CL.

Table 1. GenBank accession numbers, primer sequences, annealing temperatures and product sizes.

GenBank accession	species	primer sequence 5´–3´	T_A [°C]	product size [bp]	use
genes of interest					
*PTGS2/COX2**	*L. pardinus*	*PTGS2/COX2* Fw-1: CAC TAC AWA CTT ACC CAC TTC *PTGS2/COX2* Rv-1: AGG GGA TGC CAG TGG TAG A	55	784	a
KT119408		*PTGS2/COX2* qFw-1: CAG GAG GTC TTT GGT CTG G *PTGS2/COX2* qRv-1: CCT ATC AGT ATT AGC CTG CT	55	149	b
PTGS2/COX2	*F. catus*	*PTGS2/COX2* Fw-2: TCG ACC AGA GCA GAC AGA TG *PTGS2/COX2* Rv-2: CTG AAT CGA GGC AGT GTT GA	64	341	a
EF036473		*PTGS2/COX2* qFw-2: AAC AGG AGC ATC CAG AAT GG *PTGS2/COX2* qRv-2: GCA GCT CTG GGT CAA ACT TC	60	147	b
*PTGES**	*L. lynx*	*PTGES* Fw-1: GCT GGT CAT CAA GAT GTA CG *PTGES* Rv-1: GCT TCC CAG ACG ATC TGS A	55	339	a
KM982690		*PTGES* qFw-1: TCG CTG CCT CAG AGC CCA *PTGES* qRv-1: TAG GCC ACG GTG TGC ACC	66	153	b
PTGES	*F. catus*	*PTGES* Fw-2: ACC ATC TAC CCC TTC CTG *PTGES* Rv-2: CAG CTT CCC AGA CGA TCT	57	214	a
GU059259		*PTGES* qFw-2: GGC CTC GTT TAC TCC TTC CT *PTGES* qRv-2: CCG AAG CTT GCC CAG ATA G	60	111	b
*PTGER2**	*L. lynx*	*PTGER2* Fw-1: GTT CCA CGT GCT GGT GAC A *PTGER2* Rv-1: GAT GGC AAA GAC CCA AGG G	55	716	a
KM982691		*PTGER2* qFw-1: GAG GGG AAA GGC TGT CCA *PTGER2* qRv-1: GCA AAA ATT GTG AAA GGC AAG	56.5	103	b
*PTGER2**	*F. catus*	*PTGER2* Fw-2: AAC TAC GGC CAG TAC GTC CA *PTGER2* Rv-2: CGG GAA GAC GTT TCA TTC AT	60	356	a
EF177829		*PTGER2* qFw-2: CTT CTA CCA GCG TCG TGT CA *PTGER2* qRv-2: ACT GGC CGT AGT TCA GCA AC	60	107	b
*PTGER4**	*L. lynx*	*PTGER4* Fw-1: AGC GCT ACC TGG CCA TCA A *PTGER4* Rv-1: GAT AAG TTC AGT GTT TCA YTG G	51	830	a
KP027417		*PTGER4* qFw-1: GGG TGC CGA GAT CCA GAT *PTGER4* qRv-1: TGG TTG ATG AAC ACC CGT AC	56.5	97	b
*PTGER4**	*F. catus*	*PTGER4* Fw-2: TTG CTG TCT ATG CGT CCA AC *PTGER4* Rv-2: GGA GGT GGC GAT GAG TAA GA	61	426	a
EF177830		*PTGER4* qFw-2: TTG CTG TCT ATG CGT CCA AC *PTGER4* qRv-2: TCC AGT CGA TGA AAC ACC AG	60	102	b
*PGFS/AKR1C3**	*L. lynx*	*PGFS/AKR1C3* Fw-1: GAT GGC ACT GTG AAG AGA GA *PGFS/AKR1C3* Rv-1: CAT TCC TTC CCT GAG TTG GA	55	215	a
KM982692		*PGFS/AKR1C3* qFw-1: AGC CCG GCC TCA AGT ACA A *PGFS/AKR1C3* qRv-1: CAT TCC TTC CCT GAG TTG GA	61.5	145	b
PGFS/AKR1C3	*F. catus*	*PGFS/AKR1C3* Fw-2: CCATGGAGAAGTGCAAGGATT *PGFS/AKR1C3* Rv-2: ACC TGG AAG TTC TCC CGA AT	61	400	a
HM490147		*PGFS/AKR1C3* qFw-2: TCA ACC AGA GCA AAC TGC TG *PGFS/AKR1C3* qRv-2: CAT TCC TTC CCT GAG TTG GA	60	91	b
*PTGFR**	*L. lynx*	*PTGFR* Fw-1: CTT CAT GAC AGT GGG AAT CTT *PTGFR* Rv-1: GCT GAT GAY ATG CAC TCC AC	55	684	a
KM982687		*PTGFR* qFw-1: GCT GGA GTC CAT TTC TGG TG *PTGFR* qRv-1: CCA CGT TGC CAT TCG AAG	61	104	b
*PTGFR**	*F. catus*	*PTGFR* Fw-2: CTC TGG TCT GTG CCC ACT TT *PTGFR* Rv-2: TGA GAC CTG CCT TGT CTG TG	62	378	a
AF272340		*PTGFR* qFw-2: AGA CTT CTT TGG CCA CCT CA *PTGFR* qRv-2: GTG GGC ACA GAC CAG AGA AT	60	104	b
reference genes					
BACT	*L. pardinus*	*BACT* Fw-1: CAT CCT GAC CCT CAA GTA C *BACT* Rv-1: TCA TGA TGG AGT TGA AGG	51	625	a
KM458620		*BACT* qFw-1: GAG CAG GAG ATG GCC ACG *BACT* qRv-1: CTC GTG GAT GCC ACA GGA	62	159	b
CYC	*F. catus*	*CYC* Fw-1: CCT TCT GTA GCT CGG GTG AG *CYC* Rv-1: CTT GGA GGG GAG GTA AGG AG	56	118	a
AY029366		*CYC* qFw-1: CCT TCT GTA GCT CGG GTG AG *CYC* qRv-1: CTT GGA GGG GAG GTA AGG AG	60	118	b
GAPDH	*L. pardinus*	*GAPDH* Fw-1: CTG GTC ACC AGG GCT GCT *GAPDH* Rv-1: CCA TGA GGT CCA CCA CCC	53	640	a
KM458621		*GAPDH* qFw-1: AAG GGT GGG GCC AAG AGG *GAPDH* qRv-1: AGA GGG GCC AGG CAG TTG	63.5	143	b
RPS7	*L. pardinus*	*RPS7* Fw-1: GCC ATG TTC AGT TCG AGC G *RPS7* Rv-1: GTC TAC AAC TGA AAC TCT GGG	55	550	a
JX993349		*RPS7* qFw-1: CCT GGA GGA CTT GGT TTT CC *RPS7* qRv-1: CCT TGC CCG TGA GCT TCT	61	164	b
TBP	*L. pardinus*	*TBP* Fw-1: ATG GAT CAG AAC AAC AGC CTG *TBP* Rv-1: GCA GGA GTA CGT TAA CAG CC	56-51	970	a
JX993351		*TBP* qFw-1: AGA GAG CCC CGA ACC ACT G *TBP* qRv-1: TTC ACA TCA CAG CTC CCC AC	62.5	182	b

* gene sequence information analysed in this study; a, used for sequence analyses; b, used for expression studies; bp, base pair; Fw, forward; q, quantitative PCR; Rv, reverse; T_A, annealing temperature. Fw and Rv primers were used for sequence analysis, qFw and qRv primers were used for expression studies.

Table 2. Quantitative PCR analysis of *PTGS2/COX2* and *PTGER2* mRNA expression in different *corpora lutea* stages of the domestic cat.

relative mRNA expression [arbitrary unit]	**domestic cat**							
	pregnancy		**non-pregnant luteal phase ($p_{NP} > 0.05$)[a]**					***corpus albicans***
	preimplantation (2)	postimplantation (9)	regression (3)	formation (4)	development/maintenance (11)	early regression (7)	late regression (7)	*corpus albicans* (2)
PTGS2/COX2	0.088; 0.339	0.115 ± 0.262	0.047 ± 0.052	0.077 ± 0.068	0.027 ± 0.011	0.027 ± 0.018	0.018 ± 0.013	0.050; 0.044
PTGER2	0.028; 0.039	0.034 ± 0.019	0.021 ± 0.010	0.020 ± 0.006	0.037 ± 0.013	0.028 ± 0.012	0.029 ± 0.013	0.029; 0.058

Mean values ± standard deviation, or single values, per stage of *corpus luteum* are depicted as relative mRNA expression [arbitrary unit]. (x), n_{CL}; [a], Kruskal-Wallis rank sum test; CL, *corpus luteum*; NP, non-pregnant luteal phase.

Table 3. Quantitative PCR analysis of *PTGS2/COX2* and *PTGER2* mRNA expression in different *corpora lutea* stages of Eurasian and Iberian lynxes.

relative amount of mRNA copies [per ng total RNA]	**Eurasian lynx prooestrus**					**Iberian lynx metoestrus[a]**			
	EL1 (4) *per*	EL2 (4) *per*	EL3 (2) *per*	EL4 (4) *per*	EL5 (4) *per*	IL1 (8) *per*	IL2 (6) *per*	IL1 (3) *fresh*	IL2 (5) *fresh*
PTGS2/COX2	263.12 ± 168.20	378.43 ± 267.34	95.56; 111.13	152.86 ± 79.33	143.80 ± 45.77	60.85 ± 6.80	56.23 ± 7.74	47.84 ± 10.65	54.16 ± 25.14
PTGER2	720.22 ± 203.34	617.79 ± 196.43	336.60; 396.94	350.40 ± 88.44	759.46 ± 189.84	220.18 ± 57.09*	208.72 ± 126.94	120.74 ± 16.84*	176.48 ± 41.19

Mean values ± standard deviation, or single values, per stage of *corpus luteum* are depicted as relative amount of mRNA copies [per ng total RNA]. (x), n_{CL}; *, $p_{ILI} = 0.0121$; [a], Mann-Whitney U-test; CL, *corpus luteum*; EL, Eurasian lynx; fresh, freshly formed CL; IL, Iberian lynx; per, persistent CL.

Article 4: Siemieniuch *et al.* 2014 Mediators Inflamm

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Research Article

Placental Origin of Prostaglandin $F_{2\alpha}$ in the Domestic Cat

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In the present study, the question was addressed whether the feline placenta can synthesize prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The PGFS protein was elevated, particularly at 2.5–3 weeks of pregnancy compared to 7-8 ($P < 0.05$) and 8.5–9 weeks ($P < 0.001$). Transcripts for PGFS were significantly upregulated at 2.5–3 weeks of pregnancy and then gradually declined towards the end of gestation ($P < 0.001$). Transcripts for PTGS2 were only upregulated in placentas from queens close to term ($P < 0.001$) compared with earlier phases. Staining of PTGS2 showed distinct positive signals in placentas obtained during the last week before labor, particularly in the strongly invading trophoblast surrounding blood vessels, and also in decidual cells. Shortly after implantation, signals for PGFS were localized in the trophoblast cells. Near term, PGFS staining was seen mainly in decidual cells. Both placental $PGF_{2\alpha}$ and plasma PGFM were elevated towards the end of pregnancy ($P < 0.001$) compared with earlier weeks of pregnancy. The content of $PGF_{2\alpha}$ in extracted placenta mirrored the PGFM level in plasma of pregnant females. During late gestation there is a significant increase in PGFM levels in maternal blood and of $PGF_{2\alpha}$ levels in placental tissue concomitant with an upregulation of placental PTGS2.

1. Introduction

The domestic cat (*Felis catus*) is seasonally polyestrous, with ovulation usually provoked by coitus [1, 2]; however in as much as 30–50% of the cycles, ovulation is spontaneous [3]. During the breeding season, which extends from February-March until late summer in moderate latitudes [4], the cat exhibits several estrous cycles. If ovulation is not followed by pregnancy, the queen enters pseudopregnancy (a nonpregnant luteal phase) that lasts only about one-half of a normal gestation [5]. The length of the luteal phase in pregnant queens, accompanied by elevated progesterone (P_4) levels, is assumed to be partially driven by luteotrophic and luteolytic factors of placental origin [6]. In the dog, the uteroplacental unit seems to serve as the major source for the prepartum release of the luteolytic $PGF_{2\alpha}$ [7–9].

Even though the luteal phase in pseudopregnant cats is much shorter than in the dog, one may speculate that the feline CL possesses an intraluteal mechanism that triggers its demise at the appropriate time. The life-span of the CL in the pregnant cat is longer than in pseudopregnant queens; however, it needs to be precisely regulated to allow the onset of parturition. Although $PGF_{2\alpha}$ seems to be a very potent luteolysin, its role in regulating the CL of pregnancy in cats is still uncertain. Shille and Stabenfeldt [10] showed that high doses and repeated treatments with $PGF_{2\alpha}$ on days 11–15 after mating had almost negligible effects on progesterone levels in cats, which initially diminished but shortly afterwards recovered completely following treatment. Similarly, $PGF_{2\alpha}$ given on days 4 and 5 or 12 and 13 had no effect nor on circulating P_4 neither on CL size as monitored laparoscopically [11]. In female cats on days 21–25 after mating, luteal function was transiently depressed after treatment, but eventually the treatment did not alter the length of the luteal phase compared with untreated cats [10]. However, exogenously administered $PGF_{2\alpha}$ or its analogue has a luteolytic effect on fully developed CLs after day 33 [12] or day 40 of gestation [13].

Recently, the $PGF_{2\alpha}$ inactive metabolite 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ (PGFM), measured in the feces, was shown to be a precise pregnancy indicator in several felid species, including the domestic cat, during the last trimester of gestation [14]. The uteroplacental complex was proposed to be a source of $PGF_{2\alpha}$ in cats [14]. We hypothesize that the placenta of the domestic cat synthesizes $PGF_{2\alpha}$ in a time-dependent manner. To address this hypothesis, we examined in the feline placenta (1) the cellular localization of PGFS (AKR1C3), (2) the PGFS protein and mRNA content, (3) the cellular localization of PTGS2 and its mRNA expression, and (4) the tissue level of $PGF_{2\alpha}$ at different gestational stages. Moreover, in maternal blood plasma the $PGF_{2\alpha}$ metabolite PGFM was measured throughout gestation.

2. Materials and Methods

2.1. Animals, Tissue Sampling, and Preservation. All procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (61/2010/DTN). Thirty domestic, healthy cats, aged 1–6 years were brought into a local veterinary clinic for neutering. Ovariohysterectomy was done with the owner's request and consent. Four groups were created with queens ovariohysterectomized according to the following schedule.

(1) Postimplantation, early gestation, days 18–21 (2.5–3 weeks), $n = 8$.

(2) Mid gestation, days 28–35 (4-5 weeks), $n = 8$.

(3) Late gestation, days 49–56 (7-8 weeks), $n = 7$.

(4) Before parturition, days 60–64 (8.5–9 weeks), $n = 6$.

Additionally, samples were collected from one cat during hysterectomy around day 14 of gestation (peri-implantation period).

The day of mating, known in 18 females, was recorded as day 0. Confirmation of gestational age, whenever the day of mating was unknown, was done according to the measurements of the crown-rump length in fetuses and uterine ampullae diameters or lengths [15, 16]. In addition, measurements of the progesterone (P_4) values reported in the literature [17] and confirmed in our last report [18] were performed. Blood was drawn preanesthesia. Blood samples were collected from the cephalic vein into EDTA-containing tubes (Tyco Healthcare Group LP, Mansfield, USA) and transported to the laboratory at 4°C. Plasma obtained after blood centrifugation (3500 ×g, 10 min) was frozen at −20°C until P_4 and PGFM measurements . Direct enzyme immunoassay (EIA) was done for hormonal analysis.

Tissues were washed immediately after surgery with sterile saline to remove blood contamination then were placed into fresh sterile saline at 4°C and transported to the laboratory within 1 h. Uterine horns were slit longitudinally and pieces of uterus with attached placenta were prepared, washed in fresh saline to remove blood, preserved overnight at 4°C with RNAlater (Ambion Biotechnologie GmbH, Wiesbaden, Germany), and then stored at −80°C until total-RNA extraction. Another fragment of the same placenta was fixed in buffered 4% formaldehyde for 24 h, dehydrated, and wax-embedded. Sections (2-3 μm thick) were used to determine PGFS and PTGS2 protein localization by immunohistochemistry. Transcripts of PGFS and PTGS2 were determined using Real-Time PCR, while the content of PGFS protein was determined by western blot analysis.

2.2. RNA Isolation, Reverse Transcription, and Real-Time PCR. Total RNA was isolated from feline placenta using TRIZOL-reagent according to the manufacturer's instructions (Sigma Aldrich, Warszawa, Poland). The RNA content was measured with a Nanodrop 1000 Spectrophotometer (Thermo, USA). Prior to reverse transcription, genomic DNA contamination was removed by treatment with DNase (Sigma Aldrich, Warszawa, Poland). Reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega, Warszawa, Poland) according to the manufacturer's protocol. In brief, an experimental reaction contained 1 μg of RNA in 12 μL of reaction mix, 4 μL of reaction buffer, 2.5 μL of $MgCl_2$ (final concentration 3 mM), 1 μL of dNTP Mix (final concentration 0.5 mM each dNTP), and 1 μL of ImProm-II Reverse Transcriptase. The final volume of the RT reaction mix was 20.5 μL. The tubes were placed in a thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated at 25°C and incubated for 5 min. For the next step, tubes were incubated in a thermocycler for 1 h at 42°C. For Reverse Transcriptase inactivation, the temperature was increased to 70°C for 15 min. Samples were kept at −20°C until analyzed by Real-Time PCR.

The levels of mRNA expression of target genes were examined by Real-Time PCR using specific primers for *PGFS*, *PTGS2*, and *cyclophilin A (Cyc)*. Among several housekeeping genes that have been already examined for their stability and efficiency [Jursza, Siemieniuch, 2013, unpublished], *Cyc* was used as a housekeeping gene. All primers were purchased from Sigma Aldrich (Warszawa, Poland) and were tested in our previous report [18, 19]. The sequences were as follows: *PGFS* forward: 5′-TCAACCAGAGCAAACTGCTG-3′, *PGFS* reverse: 5′-CATTCCTTCCCTGAGTTGGA-3′ (GenBank accession number HM490147); *PTGS2* forward: 5′-AACAGGAGCATCCAGAATGG-3′; *PTGS2* reverse: 5′-GCAGCTCTGGGTCAAACTTC-3′ (GenBank accession number EF036473); *Cyc* forward: 5′-CCTTCTGTAGCTCGGGTGAG-3′; *Cyc* reverse: 5′-CTTGGAGGGGAGGTAAGGAG-3′ (GenBank accession number AY029366).

The Real-Time PCR reactions were carried out in an automated fluorometer ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) using SYBR Green Master Mix (Applied Biosystems, Applera, Warsaw, Poland). The PCR reactions were performed in 96-well plates. The total reaction volume was 20 μL containing 1 μL cDNA (200 ng), 500 nM each of forward and reverse primers, and 10 μL SYBR Green PCR Master Mix. Real-time PCR was carried out as follows: initial denaturation (10 min at 95°C), followed by 40 cycles of denaturation (15 s at 95°C), and annealing (1 min at 60°C). After each PCR reaction, melting curves were obtained by stepwise increases in temperature from 60 to 95°C to ensure single product amplification. The presence of the product was also confirmed by

electrophoresis on 2% agarose gel. Relative quantification was performed by normalizing the signals of target genes with the *Cyc* signal using the Miner method for quantifying qRT-PCR results, using calculations based on the kinetics of individual PCR reactions [20].

2.3. Protein Preparation and Western Blotting. Tissue homogenates were prepared with NET-2 lysis buffer containing 50 mM Tris-HCl, pH = 7.4, 300 mM NaCl, 0.05% NP-40, and protease inhibitor cocktail (10 μL/mL). Tissues were centrifuged at 10,000 g for 10 min at 4°C; then proteins in the supernatant were disrupted using a sonicator (75 W for 15 s). The concentrations of protein were determined by the Bradford assay with a spectrophotometer (SmartSpec Plus, Bio-Rad). Proteins were solubilized in sample buffer (25 mmol/L Tris-Cl, pH = 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue), placed onto 12% SDS-polyacrylamide gel, and separated at 120 V. Afterwards, proteins were transferred onto methanol-activated PVDF membranes for 1 h at 100 V. To block nonspecific binding, membranes were incubated for 1 h in nonfat dry milk, 5% in PBS with 0.25% Tween 20. Membranes were incubated overnight at 4°C with canine-specific guinea pig polyclonal affinity purified anti-PGFS (AKR1C3) custom-made antibody (Eurogentec S.A., Seraing, Belgium), at a dilution of 1 : 400, as described by Gram et al. [8]. After this, membranes were washed three times for 10 min in PBS with Tween-20 at 20°C. Next, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, dilution 1 : 15.000) for 1 h at room temperature (RT).

As a loading control, PVDF were reblotted with anti-β-actin mouse monoclonal antibody (sc-69879, Santa Cruz Biotechnology, Rockford, IL, USA, dilution 1 : 1000). Results were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate according to the manufacturer's protocol (Thermo Scientific, USA) and the ChemiDoc XRS+ System and Image Lab (BioRad). The optical density of bands was determined using ImageJ software.

2.4. Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were cut with a microtome (2-3 μm) and mounted on SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany). The experimental protocol was as previously described for feline placenta [18]. Briefly, slides were deparaffinized and rehydrated in a graded ethanol series and then incubated in citrate buffer (10 nM, pH 6.0) for 15 min under microwave irradiation at 560 W for antigen retrieval. Then, sections were incubated in 0.3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase and then washed in IHC-buffer/0.3% Triton X pH 7.2–7.4 (0.8 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, and 1.37 mM NaCl). Blocking of nonspecific binding sites was performed in 10% goat or rabbit serum. The following primary antibodies were used: canine-specific guinea pig polyclonal affinity purified anti-PGFS (AKR1C3) antibody (dilution 1 : 400) (Eurogentec S.A., Seraing, Belgium), the same as for western blot and as recently applied for canine uteroplacental tissue [8], and affinity purified goat anti-rat polyclonal antibody raised against the N-terminus of COX2 (Santa Cruz Biotechnology, Inc., CA, USA). The negative controls were as follows: slides without the primary antibody and slides incubated with nonimmunized guinea pig or goat serum, respectively, at the same dilution and protein concentration as primary antibodies. Sections were incubated overnight at 4°C. After washing with IHC-buffer, slides were incubated for 30 min at RT with either biotinylated goat IgG (secondary antibody, dilution 1 : 100) against guinea pig immunoglobulin (Vector Laboratories, Burlingame, US) or biotinylated rabbit IgG antibody (dilution 1 : 100) against goat immunoglobulin (Vectastain ABC Kit, Vector Laboratories). For enhancing signals, sections were incubated with the avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 30 min at RT. After washing with IHC-buffer, sections were allowed to react with the substrate diamino-benzidine (DAB; DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. Slides were counterstained with hematoxylin, rinsed under running tap water for 5 min, dehydrated in a graded ethanol series, and mounted in mounting medium DPX (Panreac Quimica Sau, Barcelona, Spain).

2.5. Placental $PGF_{2\alpha}$ Extraction. For $PGF_{2\alpha}$ extraction, the protocol elaborated by Tsang et al. [21] and tested for feline placental progesterone extraction by Siemieniuch et al. [18] was applied, with some minor modifications. Briefly, placental fragments weighing 200–220 mg were stored at −80°C. After thawing, each tissue sample was homogenized in a glass vial using a tissue disruptor with 400 μL of a Tris buffered saline containing proteins and sodium azide as preservative and acidified by addition of 45 μL 1 N HCl. After adding 3 mL of ethyl ether to the samples, they were vortexed for 10 min and incubated at −20°C for 4 h. Afterwards, the supernatant was collected and evaporated to dryness under a stream of nitrogen at 40°C. Finally, 400 μL of the Tris buffered saline was added, mixed, and allowed to sit for 15 min at 20°C. The samples were stored at −20°C until the immunoassay was run.

2.6. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 13,14-Dihydro-15-keto Prostaglandin $F_{2\alpha}$ (PGFM), and Progesterone (P_4) Determination. For $PGF_{2\alpha}$ and PGFM measurements, the commercial $PGF_{2\alpha}$ high sensitivity EIA kit (ENZO Life Sciences Inc., Farmingdale, NY, USA) and 13,14-dihydro-15-keto Prostaglandin $F_{2\alpha}$ EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA) were used, respectively, and run according to the manufacturers' instructions.

The sensitivity of the $PGF_{2\alpha}$ assay was 0.98 pg/mL. This assay is based on the competition between free $PGF_{2\alpha}$ and a $PGF_{2\alpha}$ tracer for a limited number of $PGF_{2\alpha}$-specific sheep antiserum binding sites. The cross-reactivity for various prostaglandins and their metabolites was as follows: $PGF_{2\alpha}$ 100%, $PGF_{1\alpha}$ 11.82%, PGD_2 3.62%, 6-keto-$PGF_{1\alpha}$ 1.38%, PGI_2 1.25%, and PGE_2 0.77%. The inter- and intraassay precision variation was 6.8% and 10.1%, respectively.

The sensitivity of the PGFM assay was 15 pg/mL. This assay is based on the competition between free 13,14-dihydro-15-keto $PGF_{2\alpha}$ and a 13,14-dihydro-15-keto Prostaglandin $F_{2\alpha}$

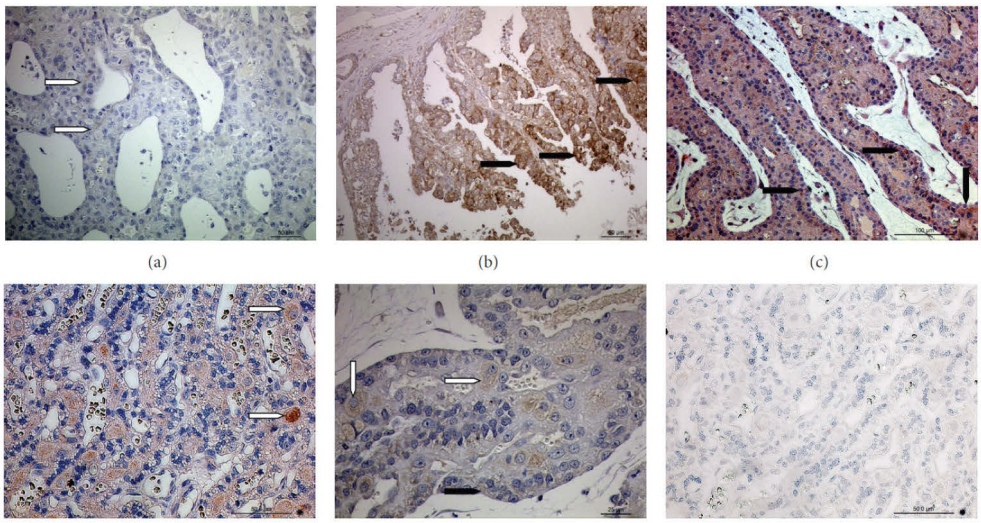

Figure 1: PGFS (AKR1C3) protein localization in the uteroplacental unit. Immunohistochemical (IHC) localization of PGFS in the week 2 of pregnant uterus (a) and placenta at week 3 (b), week 6 (c), and week 9 of gestation ((d), (e)). (a) In the feline uterus some weak PGFS signals are localized in the glandular epithelium (open arrowheads); ((b), (c)) in the placental labyrinth, PGFS expression is localized in the fetal trophoblast cells (solid arrowheads). ((d), (e)) In the fully developed placenta, towards the end of pregnancy, signals are weaker in trophoblast cells and are mostly localized in maternal decidual cells (open arrowheads). (f) Isotype control.

tracer for a limited number of 13,14-dihydro-15-keto $PGF_{2\alpha}$-specific rabbit antiserum binding sites. The cross-reactivity for various prostaglandins and their metabolites was as follows: 13,14-dihydro-15-keto $PGF_{2\alpha}$ 100%, 13,14-dihydro-15-keto PGE_2 2.7%, and 15-keto $PGF_{2\alpha}$ 1.8%. The inter- and intraassay precision variation was 8.3% and 14.5%, respectively.

For P_4 measurement, a commercial Progesterone ELISA kit (ENZO Life Science) was used and run in accordance with the manufacturer's instructions. This kit uses a polyclonal antibody to P_4 to bind, in a competitive manner, P_4 in a sample or P_4 with a covalently attached alkaline phosphatase molecule. The sensitivity of this P_4 assay was 8.57 pg/mL. The cross-reactivity for a number of related steroids was as follows: P_4 100%, 5α-Pregnane-3,20-dione 100%, 10-OH-progesterone 3.46%, corticosterone 0.77%, and deoxycorticosterone 0.28%. The inter- and intraassay precision variation was 8.1% and 9.5%, respectively.

2.7. Statistics. To test the effect of different stages of pregnancy on mRNA levels and on PGFM or $PGF_{2\alpha}$ concentrations, the Kruskal-Wallis Test (a nonparametric ANOVA) was used followed by the Newman-Keuls Multiple Comparison Test using the statistical software program GraphPad6 (GraphPad PRISM v 6.0; GraphPad Software Inc., San Diego, CA, USA). PGFM concentrations in plasma or $PGF_{2\alpha}$ concentrations in the placenta are shown as a mean ± standard deviation. Significance was defined as values of $P < 0.05$.

3. Results

3.1. Placental Expression of PGFS throughout Pregnancy. In the uterus at the peri-implantation period (2nd week of pregnancy), no or very weak signals were observed in the glandular epithelium (Figure 1(a)). After implantation (3rd week of pregnancy), and in fully developed placenta at the 7th week of gestation, strong placental signals were localized in fetal trophoblast cells (Figures 1(b) and 1(c), resp.). Towards the end of pregnancy (9th week) signals in trophoblast cells were much weaker, and were localized in the maternal decidual cells (Figures 1(d) and 1(e)). Negative controls showed no staining (Figure 1(f)).

In the western blot analysis, the PGFS was detected as a protein with a molecular size of approximately 34–36 kDa. The expression of PGFS protein was strongly affected by gestational age (Figure 2). The PGFS protein was upregulated, particularly in the first period studied, at 2.5–3 weeks of pregnancy, compared to samples collected at 7-8 gestational weeks ($P < 0.05$) or 8.5–9 weeks ($P < 0.001$).

The expression of transcripts for placental PGFS as determined by Real-Time PCR was strongly time-dependent (Figure 3(a)). The PGFS mRNA transcripts were mirrored by PGFS protein expression in western blot analysis. The PGFS mRNA was significantly upregulated at the 3rd week of pregnancy and then gradually declined towards the end of gestation ($P < 0.001$).

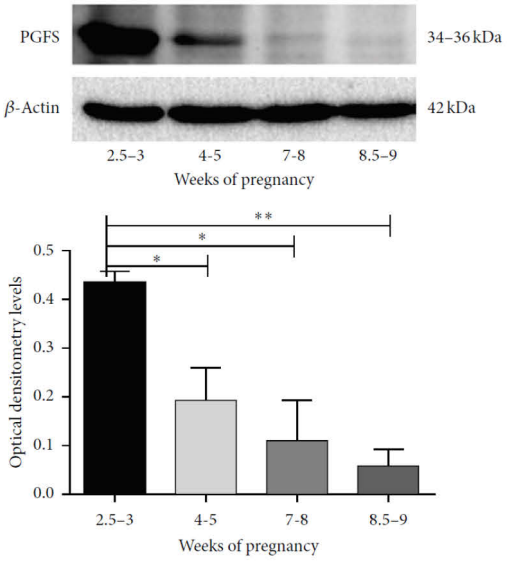

FIGURE 2: PGFS (AKR1C3) protein expression. Time-dependent expression of PGFS protein in the feline placenta. Tissue homogenates (30 μg) were used in western blot analysis (n = 16). Representative immunoblots are shown. Lower panel represents densitometric values for PGFS normalized against B-actin (mean ± S.D.).

3.2. Placental Expression of PTGS2 throughout Pregnancy. The IHC analysis revealed abundant positive signals in 9th week feline placenta. Strong staining was observed in the invading fetal trophoblast cells surrounding maternal blood vessels (Figure 4(a)). Similarly, strongly positive signals were observed in the maternal decidual cells in the 9th week of pregnancy (Figure 4(b)). No signals were noted in the negative controls (Figure 4(c)). Only weak signals were observed for placental PTGS2 protein expression during earlier stages of pregnancy.

The expression of transcripts for placental PTGS2, as determined by Real-Time PCR, was time-dependent (Figure 3(b)). PTGS2 mRNA expression was basal during early and midgestation, started to increase during late gestation, and was significantly upregulated during the prepartal phase ($P < 0.001$).

3.3. Placental Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and Blood Plasma 13,14-Dihydro-15-keto Prostaglandin $F_{2\alpha}$ (PGFM) Content. The levels of $PGF_{2\alpha}$ extracted from placentas mirrored the PGFM levels measured in peripheral blood plasma of pregnant females throughout gestation, as shown in Figures 5(a) and 5(b), respectively. Both placental $PGF_{2\alpha}$ and plasma PGFM were elevated towards the end of pregnancy compared to the values obtained at the 3rd week of pregnancy ($P < 0.001$).

4. Discussion

To examine the role of prostaglandins in the feline placenta near the end of pregnancy, the transcripts for PTGS2 and its cellular localization were analyzed. PTGS2, the crucial enzyme in generating prostaglandin, catalyzes the conversion of arachidonic acid into endoperoxide PGH_2, which is a precursor of all prostaglandins [22, 23]. In the present study, transcripts for PTGS2 were upregulated in placentas solely from queens in the last week before termination of the pregnancy. These findings are in agreement with a previous report of an acute increase of PTGS2 transcripts in the placenta in the dog during prepartum luteolysis [7]. In the present study, the protein localization of PTGS2 was found in both fetal trophoblast and maternal decidual cells. In contrast, both protein and transcripts for PGFS (AKR1C3) were strongly upregulated soon after implantation, which takes place on days 12–14 after ovulation in the cat [24]. Transcripts for PGFS and PGFS protein were rather low in the placenta near the end of pregnancy. These findings are in agreement with a report of decreased PGFS transcripts in preparturient dogs, which led to the conclusion that PGFS (AKR1C3) might be not responsible for the sharp $PGF_{2\alpha}$ elevation just before term in the bitch [7].

One of the questions addressed in this study was whether PGFS (AKR1C3), that was shown to promote the direct conversion of PGH_2 to $PGF_{2\alpha}$ [8], is responsible for placental elevation of $PGF_{2\alpha}$. In the dog, PGFS (AKR1C3) staining was predominantly localized in the fetal trophoblast cells in the fetomaternal contact zone, justifying the assumption that PGFS may play a role in decidualization and placentation; moreover, PGFS expression was low prior to implantation but distinctly increased thereafter [8]. The cellular localization of PGFS in the feline placenta from 3 weeks of gestation is similar to that observed in the dog. Therefore, PGFS is implicated in the processes of fetal membrane development and placentation. The discrepancy observed between elevated $PGF_{2\alpha}$ concentration in the placenta and downregulation of PGFS (AKR1C3) protein and mRNA levels may be explained by the number of enzymes involved in generation of the wide variety of prostaglandins. $PGF_{2\alpha}$ is one of the few primary prostaglandins derived enzymatically from the endoperoxide PGH_2. It can be directly synthesized from PGH_2 by $PGF_{2\alpha}$ synthase (PGFS), like AKR1C3 presented herein. However, other pathways of $PGF_{2\alpha}$ synthesis, including enzymes belonging to the aldo-ketoreductases family, for example, AKR1B1 or AKR1B5, are possible. The canine PGFS cDNA sequence has been cloned [25] and classified as an AKR1C3 and is the only canine-specific PGFS isoform known so far [8]. The feline PGFS cDNA sequencing was based on a dog cDNA sequence and showed 87% homology with the canine PGFS [19]. Furthermore, $PGF_{2\alpha}$ may originate from conversion of several other prostaglandins, including catalysis of PGE_2 to $PGF_{2\alpha}$ by 9-keto-reductase (9KPGR), or perhaps even at relatively low expression levels AKR1C3 is still not rate-limiting for $PGF_{2\alpha}$ synthesis.

Recently, elevated concentrations of a stable $PGF_{2\alpha}$ metabolite (PGFM) were found in the feces of several felid species in the last trimester of pregnancy [14]. In the present

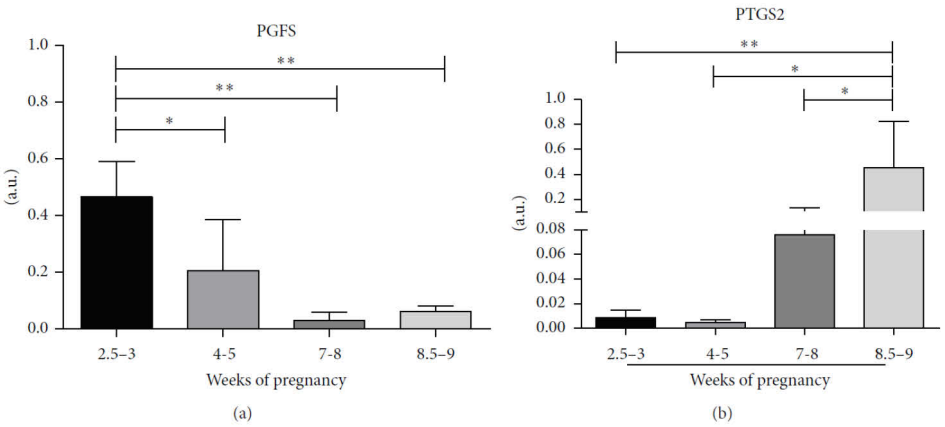

FIGURE 3: PGFS (AKR1C3) and PTGS2 mRNA expression. Time-dependent expression of feline PGFS (a) and PTGS2 (b) as determined by Real-Time PCR. Asterisks indicate statistical differences between expression levels during the course of pregnancy ($^{*}P < 0.05$, $^{**}P < 0.001$).

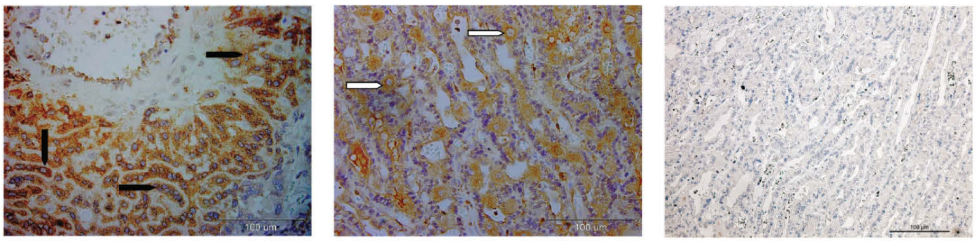

FIGURE 4: PTGS2 protein localization in the placenta. Immunohistochemical localization of PTGS2 in feline placenta from the 9th week of pregnancy. Positive signals are shown in the strongly invading trophoblast surrounding blood vessels (solid arrowheads) (a) and also in maternal decidual cells (open arrowheads) (b). (c) Isotype control.

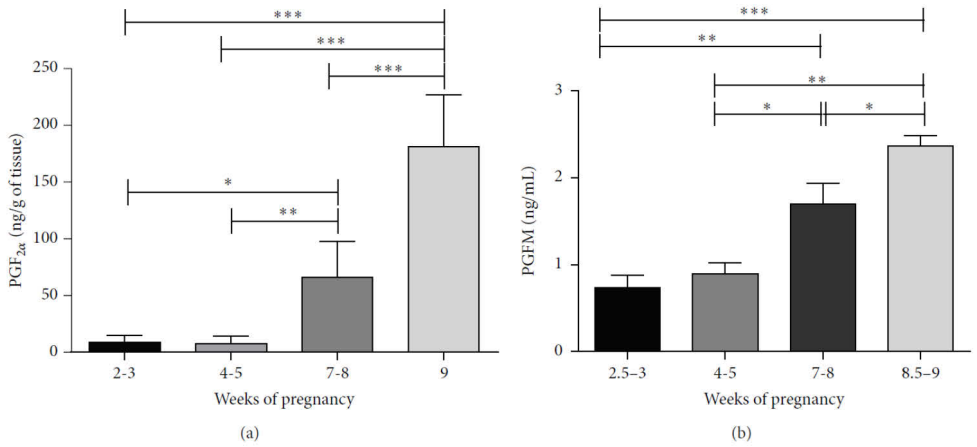

FIGURE 5: Placental $PGF_{2\alpha}$ and serum PGFM values. Time-dependent content of $PGF_{2\alpha}$ in placenta (a) and PGFM in blood plasma (b) as determined by immunoassays. Asterisks indicate statistical differences between prostaglandin levels during the course of pregnancy ($^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$).

study, the PGFM level, measured in feline plasma blood collected just before ovariohysterectomy, was low during the first and second trimesters of pregnancy and then started to increase for the last 3 weeks of gestation. Certainly, PGFM in the maternal plasma may not originate exclusively from the placenta. Nevertheless, to examine whether the placenta might support synthesis of $PGF_{2\alpha}$, placental tissues from different gestational weeks were extracted to obtain $PGF_{2\alpha}$. The PGFM blood plasma level was mirrored by the placental $PGF_{2\alpha}$ concentration. The lowest amount of $PGF_{2\alpha}$ was observed in tissue collected soon after implantation, indicating there is no likely biological role at this time. It explains why in the earlier studies by Wildt and coworkers [11] or Shille and Stabenfeldt [10] $PGF_{2\alpha}$ failed to influence P_4 production or reduce CL size. The first rise in concentration was seen at the beginning of the second trimester, but the most distinct increase coincided with the last week of gestation. The very similar pattern of placental $PGF_{2\alpha}$ and its stable metabolite in feline blood plasma may suggest the placental origin of PGFM. However, this finding does not rule out an intraluteal and/or uterine source of $PGF_{2\alpha}$ during pregnancy, especially since components of the underlying synthetic pathways were already found in the domestic cat CL (Zschockelt and Siemieniuch 2013, unpublished). This is in contrast with the canine CL, in which intraluteal prostaglandin synthesis is associated with luteal formation but not with luteal regression or luteolysis [8, 26, 27]. The strong prepartum increase in circulating $PGF_{2\alpha}$ observed in the dog seems to be originated from the uteroplacental compartment, where strongly upregulated expression of the prostaglandin system was reported [7] and was evidenced by the increased output of $PGF_{2\alpha}$ from the canine placenta at the time of parturition [9]. Furthermore, the prepartum increase in peripheral $PGF_{2\alpha}$ in the dog is accompanied by a steep P_4 decline, implicating its role during luteolysis and/or fetal expulsion [28, 29]. These results partially agree with our present observations concerning elevated PGFM levels in maternal blood in the domestic cat and are consistent with the previous report from our laboratory presenting a considerable decline of P_4 in both maternal plasma and placental tissue in the last week of feline pregnancy [18]. The latter observation, however, concerning the P_4 levels does not relate to the immediate prepartum time period but describes the strongly diminished P_4 levels during the last gestational week [18]. At least, the luteolytic role of $PGF_{2\alpha}$ in the second half of feline gestation is generally accepted, since $PGF_{2\alpha}$ or its analogues given to females with fully developed CLs induces luteolysis and consequent abortion after day 33 [12] or day 40 of gestation [13].

In conclusion, the data presented here confirms that the placenta of the domestic cat is capable of synthesizing $PGF_{2\alpha}$ in a time-dependent manner. Besides the fact that AKR1C3 is not a rate limiting enzyme even at relatively low expression levels, it may still be determinant for $PGF_{2\alpha}$ synthesis in the feline placenta. However, other enzymes and other synthetic pathways must also be taken into account. The peak of both placental $PGF_{2\alpha}$ and maternal plasma PGFM coincides with the beginning of the last week of pregnancy in cats, compared to dogs, in which peak plasma PGFM is observed 24–48 h before term. Other pathways involved in $PGF_{2\alpha}$ synthesis in the cat placenta are possible and need to be elucidated.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Marta J. Siemieniuch conceived and designed the study, participated in molecular and hormonal analysis, and drafted the paper. Mariusz P. Kowalewski participated in design of the study and interpretation of the results and assisted in drafting the paper. Ewelina Jursza carried out Western Blot analysis. Anna Z. Szóstek participated in immunoassay procedures. Lina Zschockelt carried out gene analysis. Alois Boos assisted in drafting the paper. All authors read and approved the paper.

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